Targeting cancer through tumor-selective mRNA stabilization

Thesis

Targeting Cancer through Tumor-selective mRNA Stabilization

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This thesis is dedicated to my parents and my wife, whose courage, love and patience have been an inspiration to me.

Abstract

The success of cancer gene therapy is hindered by various physiological barriers to therapeutic vector transport from the site of injection to the nucleus of all the tumor cells. The application of replicating viruses for the treatment of cancers can overcome this problem. But this approach is limited by normal tissue tolerance of toxicity determined by local concentration of transgene products and viral proteins. Major improvements in vector targeting technology are required before any clinical success. On this basis, this thesis tests the hypothesis to target transformed tumor cells by using a novel post-translational mRNA stabilizing mechanism, which is occasionally deregulated in cancer. The overexpression of various proteins associated with rapid responses to inflammation and/or proliferation can be controlled at the level of mRNA stability. Since tumor cells continually recapitulate intracellular programs of proliferation, we hypothesize that we can use the tumor cell selective stabilization of mRNA as a novel means to target different malignant diseases.

Cyclooxygenase (COX) is the key enzyme in the conversion of arachidonic acid to prostaglandin during inflammation and many studies have linked elevated expression of COX-2 to the pathology of breast, colorectal, head and neck and other types of cancer. It has been shown that the up-regulation of COX-2 is a downstream effect of RAS-mediated transformation. Although the COX-2 over-expression in cancer is associated with increased transcription of the COX-2 gene, a large component of RAS-induced up-regulation is also mediated by a selective stabilization of the mRNA of the COX-2 gene in RAS-transformed cells. In this project, we show tumor selective mRNA stability via COX-2 3' UTR by fusing it with the adenovirus early essential gene E1A, thereby

obtaining a conditionally replicating adenovirus vector which will preferentially replicate in the RAS transformed cells.

There are wide range of genes reported in the literature with 3'UTR, which confers destabilized activity on their cognate mRNA, but whose actions are reversed under certain physiological conditions. These include hypoxia responsive 3'UTR, radiation responsive elements and 3' UTR, which mediate increased mRNA stability in proliferating cells. Therefore, the linkage to 3'UTRs is a general strategy that could be used to confer tumor cell specificity to expression of therapeutic and/or replicative genes in a wide variety of vectors and to target specific physiological situations within tumors.

Acknowledgements

This thesis is the result of a three and half years long journey whereby I have been accompanied and supported by many people. It is with pleasure that I have now the opportunity to express my gratitude to all of them. This is an impossible task, given the many people that have helped me to reach this milestone. I am going to try anyway, and if your name is not listed, rest assured that my gratitude is not less than for those listed below.

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Chapter 1

Introduction

1.1 Gene therapy

1.1.1 Introduction to gene therapy

The basic principle of gene therapy is to introduce a functional gene into a diseased target cell and restore the normal physiological function of that gene in order to revert the disease state or slowdown the progression of the disease. Over the years, a large number of inherited and acquired diseases have been targeted by gene therapy to provide new therapy. But success is considerably limited by the inability to develop a safe and effective gene transfer vehicle with which to transport genetic material into the target cells. The first major success in the field of gene therapy was the retrovirus-based therapy for infants suffering from the X-linked severe combined immune deficiency (SCID-X1) and showed real promise for long-term and even permanent cure of hereditary diseases (Cavazzana-Calvo et al., 2000). However, the field suffered serious setbacks from recent findings that two of the SCID-X1-treated patients developed a leukemia-like condition due to the vector-mediated insertional mutagenesis (Hacein-Bey-Abina et al., 2003). It has rapidly become obvious that major improvements are required in all aspects of gene delivery vector development and targeting of gene expression in order to treat any disease successfully using gene therapy. Also, various features of each vector and type of disease need to be defined before decisions are made as to which vector should be applied. The purpose of this part of the chapter is to describe the development of the field of gene therapy by discussing the history, the problems and the premise of this field.

1.1.2 A brief history of human gene therapy.

The concept that genes can be used to treat human diseases goes back several decades. In the early 60s, when the fundamentals of molecular genetics and gene transfer technology

in bacteria were established, gene transfer into animal and human became inevitable. But it wasn't until the discovery of reverse transcriptase and sequence-specific restriction endonucleases in the late 60s and 70s that gene therapy became a reality. In this part of the chapter, I will briefly review the revolution of molecular genetics and technology that gave birth to the field of human gene therapy.

1.1.2.1 The early development of gene therapy

The concept of gene therapy came before the molecular genetics revolution, beginning with the discovery by Oswald Avery and his colleagues that a gene could be transferred within nucleic acids during the Second World War (Avery and McCarthy 1944). They were able to demonstrate that transferring genomic DNA from one strain of bacteria to another changes the recipient's phenotypes into that of the donor's. The ability of viruses to transmit genes was first demonstrated in *Salmonella* by Zinder and Lederberg, which is thought to be a critical point of reference in terms of the development of gene transfer technology (Zinder & Lederberg, 1952). Then Elizabeth Szybalska and Wadaw Szybalski performed the earliest mammalian gene transfer experiments, where they were able to transform mammalian cells with a foreign DNA (Szybalska & Szybalski, 1962). In 1968, researchers from Salk Institute were able to immortalize mammalian cells by SV40 viral DNA and demonstrated the integration of proviral DNA into the host genome (Sambrook et al., 1968) . The ability of virus to transfer foreign genetic materials in the target cells and the fact that such genetic information can be stably expressed sparked the idea of treating genetic diseases by substituting a normal gene for a defective one.

1.1.2.2 Virus as a vehicle of gene transfer

In the late 1960s, Stanfield Rogers and his research group in the Oak Ridge National Laboratory in Tennessee were working with the Shope papiloma virus, which had long been known to cause warts in rabbits when applied topically to rabbit skin. In the late 1950s, Rogers's group reported that Shope papilloma virus infected rabbits skin cells contained high levels of active arginase (Rogers, 1959). Because no detectable arginase activity was observed in the normal rabbit skin, they concluded that the virus genome contained an arginase gene, which was introduced in the skin cells by transduction. At the same time several other investigators had demonstrated that the arginase found in the Shope papilloma virus infected rabbit skin cells showed very similar physical and chemical properties of the arginase found in the normal rabbit liver (Satoh & Ito, 1968). These observations were contradictory to Rogers's conclusions about the Shope papilloma virus-mediated arginase induction and were somehow overlooked by his group. However, one reproducible observation made by Roger's group was the decreased serum arginine level after the systemic administration of Shope papilloma virus in rabbits. In 1966, they also reported that researchers who worked with the Shope papilloma virus in Roger's laboratory had prolonged decreased levels of serum arginine. Based on these observations, in 1970s Rogers and his colleagues had become involved in testing the Shope gene transfer model for clinical application to cure hyperargininemia, a human disease caused by a defect in urea cycle in the liver and characterized by elevated serum arginine levels and deficiency in cellular arginase enzyme activity (Terheggen et al., 1972). Large amounts of purified Shope papilloma virus were injected systemically into two sick children with hyperargininemia (New York Times, 20 September 1970). The clinical argument behind

this premature and probably very first human gene therapy clinical trial was no alternative treatment available at that time for hyperargininemia and aggressive new approaches were the only way to help those patients (Rogers & Pfuderer, 1968) (Rogers, 1968). Several years later, in their final publication on hyperargininemia gene therapy study, Rogers and his collogues reported that the systemic administration of the Shope papilloma virus into the patients with hyperargininemia did not reduce the serum arginine levels or alter the course of the disease (Terheggen et al., 1975).

The scientific community heavily criticized this clinical study at that time. It is not difficult for us to understand why the hyperargininemia clinical trial failed. Rogers's group neither had enough information about the Shope papilloma virus, nor they had any clear ideas about the source and the mechanism of the Shope papilloma virus-induced arginase activity. Now the Shope papilloma virus genome has been sequenced (Giri et al., 1985) and we know that viral genome does not contain any arginine gene. There is no doubt that these experiments were attempted too early, before the necessary technologies were developed. But one should remember Rogers's attempt to cure hyperargininemia as we commemorate the Wright brothers' innovation. It was crude, but taught us how to fly, inspired us to explore space. As Theodore Friedmann said (Friedmann, 2001), "perhaps the most interesting part of this history, even in the face of the flawed design and failure of the clinical study, was Rogers's insight into the potential use of viruses as vectors to add new genetic information into human cells for therapy."

1.1.2.3 The development of viral vector for gene therapy

The controversies surrounding the first human gene therapy clinical trial by Rogers's group concealed their groundbreaking attempt to produce one of the earliest viral vector for

gene therapy (Jackson et al., 1972). The tobacco mosaic virus (TMV), which causes diseases in tobacco plants was one of the best understood eukaryotic viruses at that time and all the assays required for studying this virus were readily available. Rogers and his colleagues elected to chemically modify the RNA genome of the TMV virus and to show that in Jackson's word, " sequences of nucleotides can be added to a virus RNA *in vitro* and the virus used as a vector to transmit the desired information". They were able to add poly (A) sequences to purified TMV RNA genome by using the polynucleotide phosphorylase and reported that plants infected with this modified virus contained elevated levels of tetra-lysine and penta-lysine oligomers, indicating expression of the modified poly(A) added to viral genome.

In the early 1970s, Paul Berg and his group from Stanford University foresaw the potential of using virus to introduce the corrective genetic materials into victims of genetic diseases and developed the first viral vector system based upon the rhesus polyoma virus (SV40). They were able to introduce new genetic information such as the λ phage DNA and the galactose operon gene from *E. coli* into the SV40 DNA (Jackson et al., 1972). But the alarming ability of the SV40 virus to transform cells in the culture and cause cancer in rodent delayed the testing of these vectors (Watson, DNA 2003). In 1976, Burg was able to show that the recombinant SV40 vectors carrying lambda phage DNA were able to propagate in cultured kidney cells (Goff & Berg, 1976). In the late 1970s, the reverse transcriptase enzyme was discovered and Berg and his colleagues were able to show that *in vitro* synthesis of rabbit globin cDNA. Then they exchanged the globin cDNA with the major capsid protein, VP1 of the SV40 virus and showed that cells infected with this recombinant virus expressed the rabbit-globin protein (Mulligan et al., 1979). Both Roger's

and Berg's experiments were milestones for the development of viral vectors in gene therapy.

1.1.2.4 Early gene transfer studies in animals and human

In 1980, the head of the department of hematology at UCLA, Martin Cline and his group successfully transferred the DHFR gene by using calcium phosphate transfection into mouse bone marrow cells *in vitro*. Then they transplanted the modified cells into irradiated mice and showed that the recipient animals had an increased percentage of donor marrow cells with elevated DFHR enzymatic activity (Cline et al., 1980). Based on this experimental data, Cline and his colleagues transferred the β-globin gene in human bone marrow with a calcium phosphate transfection method, which was then transplanted back into the thalassaemia patients in Italy and Israel (Beutler, 2001). Although the patients suffered no adverse effects from the therapy, this clinical trial was criticized for both the scientific and the procedural design. Cline was disciplined by his home institution and by NIH (Wade, 1981) for breaking the institution guidelines, therefore, NIH established a new rule for all new gene therapy trials to be approved by the Recombinant DNA Advisory Committee (RAC).

In the early 1980s, two other successful gene transfer experiments in animal models are worth mentioning in this overview. In the first study carried out by Rubin et al., where they transferred a normal xanthine dehydrogenase gene, which is responsible for wild-type eye color in *Drosophila*, into embryos containing the defective gene by using transposon and were able to restore the wild-type eye color (Rubin et al., 1982). The second experiment was a retrovirus-mediated growth factor transferred into transgenic dwarf mice, which mimicked human pituitary dwarfism and resulted in the correction of murine dwarfism

(Hammer et al., 1984). But the mice grew 50% larger than the normal size due the deregulated expression of the corrective gene.

1.1.2.5 Gene therapy begins to come of age

Steven Rosenberg and French Anderson did the first approved clinical trial for human gene therapy on May 22, 1989, where they used a retrovirus to transduce the tumor-infiltrating lymphocytes (TIL) with the neomycin resistance gene as a marker for the infused cells (Morecki et al., 1991). The aim of this trial was not therapy, but to evaluate the applicability and side effects of this method. Five patients received the gene-modified TIL. The presence and expression of the neomycin-resistance gene were detected in TIL from all the patients with southern blot and cells from four out of five patients grew successfully in high concentration of a neomycin analogue, G418. This study was able to demonstrate the safety of the retroviral vector-mediated gene transduction for human gene therapy. The first RAC-approved human gene therapy trial with a therapeutic aim began 18 month later. This time, Anderson and his colleagues at NIH attempted to cure severe combined immune deficiency (SCID), a monogenic disease caused by the lack of the enzyme adenosine deaminase (ADA). They were able to insert an ADA gene into T cells of two children suffering from SCID (Blaese et al., 1995). Although the infusion of corrective gene-modified T cells did not fully reverse the disease symptoms, it did significantly reduce the amount of the drug PGE-ADA needed to treat them (Blaese et al., 1995).

1.1.3 Viral Vectors for Gene Therapy

Viruses have evolved for thousands of years to become a biological machine that efficiently gains access to host cells and hijacks the cellular machinery to support their replication. The idea behind virus-based vectors for gene therapy application utilizes the

viral infection pathways to deliver desired genetic information, but avoids the subsequent expression of viral genes, which leads to viral replication and toxicity. This is achieved by replacing all, or some, of the coding regions from the viral genome with the genetic information of a desired therapeutic gene, but leaving intact those genes that are required for the packaging of vector genome with the therapeutic gene into the viral capsid. The deleted genes encoding proteins are usually essential for virus replication or capsid/envelope proteins, which are included in a separate construct in the packaging/producer cells to provide helper function *in trans*. The recombinant nonreplication vector particles carrying a therapeutic gene can be produced by introducing the modified vector sequence into the producer cells (**Figure 1.1**). The ability to insert desired genetic information into a replication defective viral vector is the backbone of developing virus-based gene transfer technology.

All the currently available viral vectors for gene therapy are based on the different which viruses can be categorized into two groups: I) integrating vectors and II) nonintegrating vectors (Verma & Weitzman, 2005) Vectors based on oncoretrovirus, lentivirus and adeno-associated virus can integrate packaged sequences into the host cell chromosomal DNA and maintain lifelong gene expression. Adenovirus and herpes simplex virus based vectors are nonintegrating vectors. The packaged genetic information delivered by these vectors remains episomal in the nucleus of the target cells. In this part of the chapter, I will give a brief overview of the development of all the major gene therapy vectors derived from different viruses.

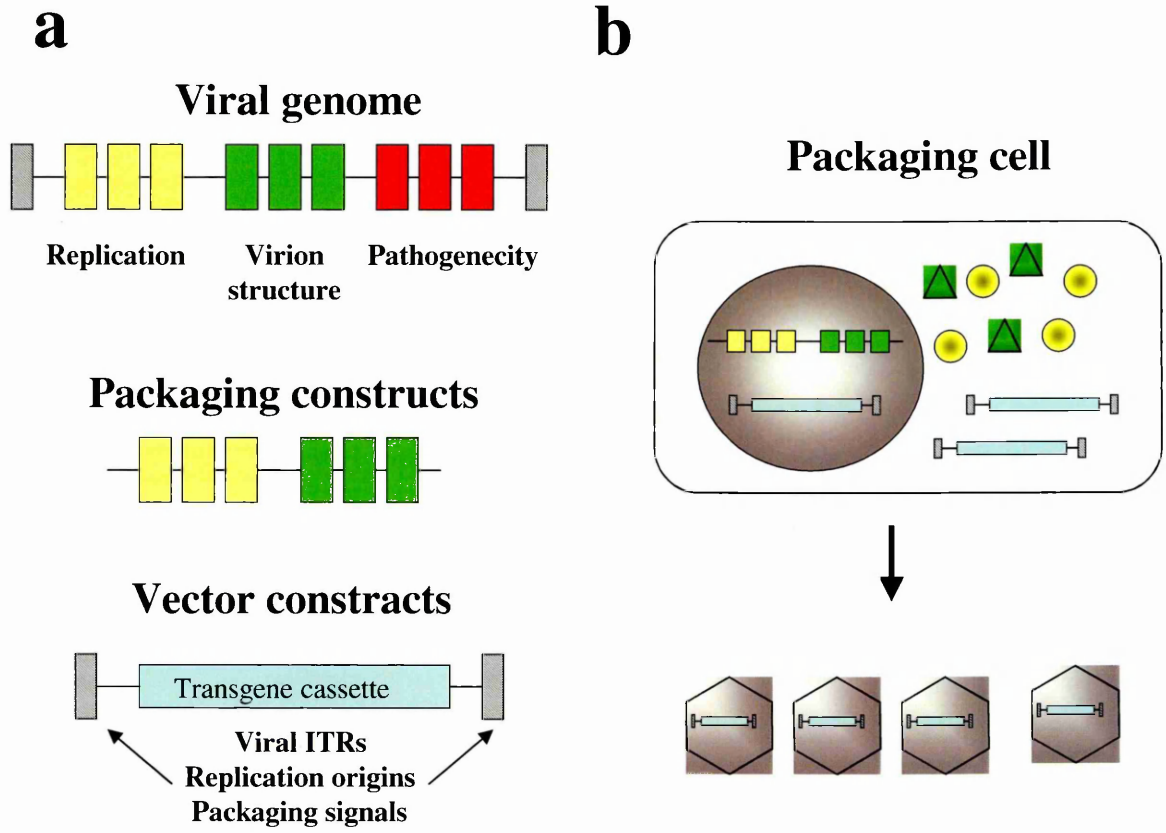


Figure 1.1 Principle of generating a viral vector for gene therapy. (a) Different viral genes that are involved in replication, production of the virion, and pathogenicity after viral infection. The packaging constract contains only genes that requires for replication and structural proteins. The vector construct contains the essential cis-acting packaging signals and the transgene cassette that contains the therapeutic gene. (b) The packing and vector constructs are introduced into the packaging cells by transfection, which stably expressed proteins required for replication and assembly for the recombinant vectors. Adapted from Kootstra (2003).

1.1.3.1 Retrovirus

Retroviruses are a large family of enveloped viruses, which contain two copies of the RNA viral genome flanked by 5' and 3' terminal repeats (LTR) (Pages & Bru, 2004). The RNA genome holds three essential genes: the *gag* gene that encodes for the core proteins

capsid, matrix and nucleocapsid; the *pol* gene which encodes for the viral enzymes protease, reverse transcriptase and integrase; and the *env* gene encodes for envelope glycoproteins, which mediate virus entry. After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion and the RNA genome converts into double-stranded proviral DNA by the viral enzyme reverse transcriptase. The proviral DNA then translocates to the nucleus with the preintegration complex during mitosis and integrates into the host cell genome by the viral integrase.

Retrovirus based vectors are amongst the earliest and most widely used viral vectors for gene therapy (Shimotohno & Temin, 1981). These vectors were initially based on gamma retrovirus genus, mainly Moloney murine leukaemia virus (MoMuLV). To generate recombinant retroviral vectors, the *gag, pol* and *env* genes are replaced by the cDNA of therapeutic gene up to 6-7 kb in a vector, which only contains the packaging signals and two LTRs. All three deleted viral genes are constitutively expressed in a cell line known as packaging cell, which provides the necessary helper function for the propagation and production of retroviral vector. Because these vectors are capable of integrating the therapeutic gene into the host genome, they are the ideal vectors for the long-term gene expression to correct monogenic diseases. But the main concern in using retroviral vector is the insertional mutagenesis caused by accidental random integration into the host chromosome resulting in the activation of certain protooncogenes. Another limitation is the proviral integration and gene expression required for active cell division (Verma & Weitzman, 2005).

1.1.3.2 Lentiviruses

Lentiviruses are members of retrovirus family, which encode three to six more viral proteins in addition to *gag, pol* and *env* (Kootstra & Verma, 2003). I will focus on the human immunodeficiency virus type 1 (HIV-1)-based vectors because, they have been most extensively used for gene therapy. In addition of all the advantages described above, which are common to all retroviral vectors, vectors derived from lentiviruses offer one great advantage over their oncoretroviral counterparts: they can transduce nondividing cells, an important requirement for genetically modifying tissues for potential targets for gene therapy. Vectors derived from HIV-1 allow for the efficient in vivo delivery, integration, and stable expression of transgenes into cells such as neurons, hepatocytes and myocytes (Blomer et al., 1997, Kafri et al., 1997)). But the safety of HIV-based vectors requires a most careful evaluation, considering the pathogenicity of the parental virus.

HIV-1 encodes six accessory proteins (*Tat, Rev, Vif, Vpr, Nef,* and *Vpu*). Using a similar strategy that is used for the production of retroviral vector can generate the HIV-based vector. The third-generation packaging unit of HIV-1-based vectors conserves only three of the nine genes present in the genome of the parental virus, which eliminates possibility of reconstitution of a wild-type virus through recombination. These vectors are deleted for all the viral genes except the LTRs, the packaging signal and the *Rev* responsive element (RRE). The *Rev* proteins, if provided *in trans* during vector production, ensure efficient nuclear export of the viral RNA through binding to the RRE. Initially, the vector RNA was derived by the endogenous LTR promoter, but the next generation HIV-based vector utilizes a CMV/LTR hybrid promoter to make the vector *Tat*-independent. The presence of the nuclear target signals in the viral accessory protein *Vpr* allows the

integration of the viral/therapeutic genome into the host genome in both dividing and non-dividing cells making them an attractive vector system. However, the safety of the HIV-based vectors in the clinical setting is a major concern. This problem is addressed by the recent development of self-inactivated vectors, where the 3' LTR is partially deleted to prevent mobilization following infection with HIV-based vector (Miyoshi et al., 1998) and minimize the risk of insertional mutagenesis.

1.1.3.3 Adeno-associated virus (AAV)

AAV is a small, no enveloped, nonpathogenic DNA virus that belongs to the Parvoviridae family (Kootstra & Verma, 2003). The viral genome is a linear, 4580 base pairs single-stranded DNA (ssDNA), which is inserted between two T-shaped ITRs carrying two major viral open reading frames (ORF). The *cap* ORF encodes for the structural proteins that form capsid, whereas the *Rep* ORF produces the regulatory proteins. After the virus enters the cell, the ssDNA is converted into the double-stranded DNA and is directed to host chromosome by *Rep* proteins, where it integrates by nonhomologous recombination. Successful AAV replication requires coinfection with a helper virus such as adenovirus or herpes virus. To generate recombinant AAV vector (rAAV), the cDNA of a therapeutic gene is inserted between the two AAV ITRs in an expression plasmid. The second plasmid is the helper plasmid that provides all the necessary AAV proteins like *Cap* and *Rev* in trans. These two plasmids are cotransfected in a permissive cell line such as 293 followed by helper adenovirus infection. Most recombinant AAV vectors have been derived from serotype 2 capsid (Carter & Samulski, 2000). But, so far, a total of eight different AAV serotypes have been identified that utilize different cellular receptors for cell entry, which give each serotype a unique tropism (Grimm & Kay, 2003). Pesudotyping the

rAAV2 with capsids from other serotypes to achieve more efficient gene transfer in targeted tissues is becoming a common strategy (Grimm & Kay, 2003). One of the major problems of using the AAV vectors for gene therapy is contamination with the wild type AAV and helper virus in purified rAAV stocks. However, new vector systems and packaging cell lines have been designed to overcome these problems.

1.1.3.4 Herpes Simplex Virus

Human herpesviruses are a class of large double-stranded DNA viruses with the ability to accommodate a large amount of foreign DNA (Epstein et al., 2005). The viral genome is about 152 kb in size and is divided into unique long (U_L) and unique short (U_S) regions that are flanked by terminal repeats. The virus encodes at least 80 viral proteins with very little gene splicing. Natural herpes virus infection can be lytic in epithelial cells or persist in a latent state in the neuronal cells. All of the gene therapy vectors are derived from type 1 herpes simplex virus. Two different strategies have been used to generate recombinant viral vectors. The first strategy uses the replication defective HSV-1 vectors contained deletion of all, or the five immediate early genes (ICP0, ICP4, ICP22, ICP27 and ICP47) that are responsible for lytic infection (Berto et al., 2005). They can carry large transgenes up to 30 kb in size and can be produced in high titers by using complementary cell lines that provide the deleted early genes *in trans*. But these vectors still contain large proportions of wild type HSV-1 genome and can express many different viral genes, which induce cytotoxicity and immune responses against the therapeutic vector. The second HSV vector system is known as the HSV-1 amplicon vector system, which is based on the ability of HSV-1 to package defective genomes carrying the *cis*-acting sequences *ori* (origin of viral DNA replication) and *pac* (packaging and cleavage signal). Beside the *cis*-acting sequences, all

other wild type viral genes are deleted from the amplicon vectors. For this reason, packaging and production of the amplicon vectors require a replicating helper virus infection, which can result in high-level contamination with the helper virus. This problem is overcome by the development of a bacterial artificial chromosome carrying all the viral genes without the *pac* signal. The HSV vector systems have been applied to gene therapy for multiple diseases, including brain tumors, neurological diseases and spinal nerve diseases. The major limitation for recombinant HSV-1 vector is the host immune response. But the large packaging capacity of HSV-1 based vectors may be useful for delivering complex genes.

1.1.4 Adenovirus

Half a century ago Rowe and his colleagues first isolated adenovirus from culture adenoid tissue in the laboratory (Rowe et al., 1953). Since then, this virus has been used as a powerful model system to study basic cellular processes such as transcription, RNA processing, DNA replication, cell cycle and oncogenesis. In some earlier studies, it was observed that adenovirus could recombine in tissue culture setting (Lewis & Rowe, 1970) and that became the foundation for the use of the adenovirus as a vector for gene therapy. In this part of this chapter, I will briefly discuss the structure and life cycle for the human adenovirus and then give an overview of the use of adenovirus vectors as a gene therapy vehicle.

1.1.4.1 Adenovirus structure

Adenovirus is a nonenveloped icosahedral particle about 70-90 nm in size with a viral capsid that surrounds the viral core containing the large double-stranded DNA genome of

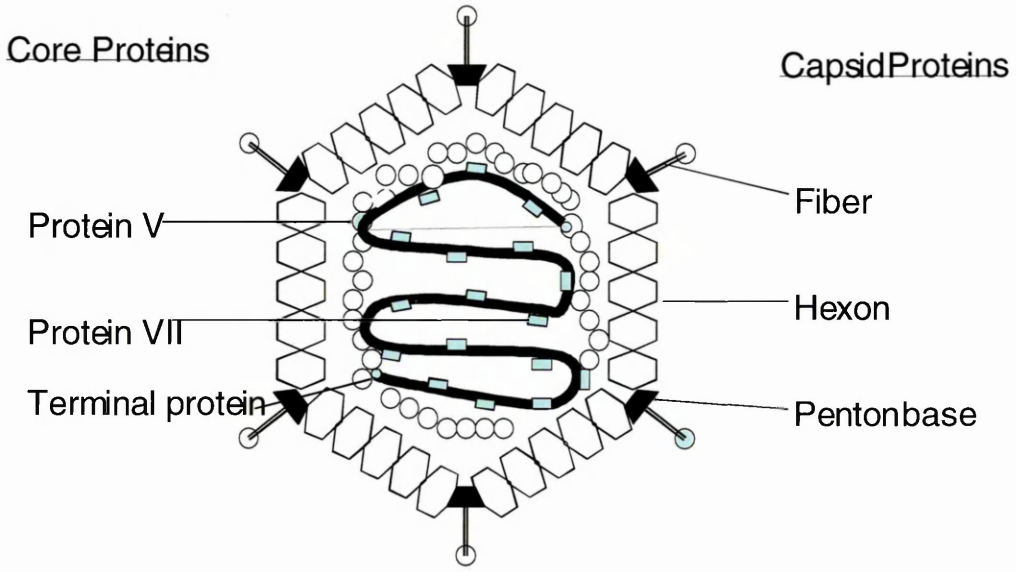


Figure 1.2 Schematic of the adenovirus particle, showing major component of the capsid and the core. Adapted from Shenk (1996).

36 kb (**Figure 1.2**) (Berk, 2005). The capsid is made of three types of proteins: the hexon proteins, which form homotrimers and 240 of these hexomers form the basis of the icosahedral structure; the fiber form trimers which is associated with each of the 12 penton vertices and is responsible for the initial attachment of virions to the cell surface; and the penton base which form 12 pentomers that anchors the fiber. So far, at least 51 distinct serotypes of human adenoviruses have been isolated and classified into six groups (A-F) based on the sequence homology and their ability to agglutinate red blood cells.

1.1.4.2 Adenovirus binding and entry

Except for the group B adenoviruses, the initial attachment of all other groups to the cell surface occurs through binding of the fiber knob to the Coxsackie and Adenovirus Receptor (CAR) (Coyne & Bergelson, 2005). CAR is a type 1 transmembrane protein in the immunoglobulin superfamily, which normally functions as a cell-to-cell adhesion molecule (Honda et al., 2000) and is expressed in many human tissues including heart, lung, liver and brain (Howitt et al., 2003). After the initial binding to CAR, the RGD motif

on the penton base interacts with the $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrin molecules and triggers virus internalization by the clathrin-dependent, receptor-mediated endocytosis (Meier et al., 2002).Then the virions escape from the endosome to cytoplasm by an unknown mechanism, traffic toward the nucleus by a dynein-mediated, microtubles dependent migration where they subsequently dock with the Nuclear Pore Complex (NCP) (Trotman et al., 2001). The viral capsid disassembles at the NPC, transports the viral genome in the nucleus and initiates the viral transcriptional program.

1.1.4.3 Adenovirus genome and replication

Adenovirus genes can be divided into three major groups depending on the time course of their gene expression during the viral replicative cycle: early (E1A, E1B, E2, E3, and E4), delayed (IX and Iva2), and the late transcription unit. (**Figure 1.3**). E1A is the first viral transcription unit that is expressed after infection, which then *trans*-activates the other adenovirus early genes and pushes the infected cells to enter S phase by sequestering the

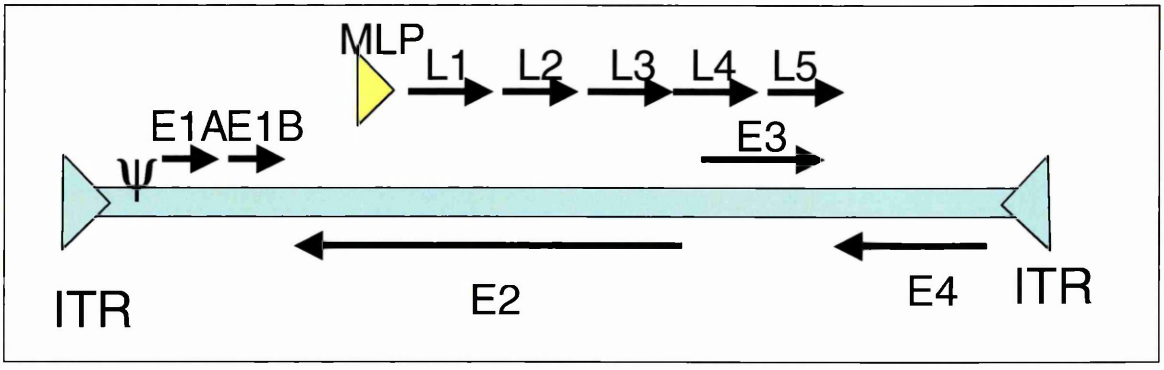


Figure 1.3 Map of the adenovirus genome and transcription unit. Position of the left and right ITRs, the packing sequence (ψ), the early transcription units (E1, E2, E3 and E4) and the major late transcription unit (MLP, L1-L5) are shown. Arrows indicate the direction of transcription. Adapted from Mcconnell (2004).

Rb proteins and subsequently releasing the E2F transcription factor in order to create an optimal environment for virus replication (McConnell & Imperiale, 2004). But this E1A mediated cell cycle deregulation results in an accumulation of the tumor suppressor p53 and the activation of p53-dependent apoptosis pathway, which prevents the survival of infected cells. The adenoviral E1B-55k protein and the E4 region product E4orf6 together block the p53-dependent apoptosis by directly binding p53 and inhibiting its transcription activity to express proapoptotic genes (Sarnow et al., 1982). The E2 region contains DNA polymerase, preterminal protein (pTP), and the 72-kDa DNA-binding protein, which are necessary for the replication of the viral DNA. The pTP acts as a telomere to maintain the integrity of the viral DNA. The products of the viral E3 region suppress the host immune response by interfering with the antigen processing and presentation in order to allow the virus to replicate more efficiently. The E4 gene products have been known to play a role in cell cycle control and transformation; however, the mechanisms underlying these functions are not well understood.

The adenoviral major late promoter (MLP) derived most of the late genes. This major late transcription unit encodes approximately 15 to 20 different mRNAs, all of which are derived from a single pre-mRNA by differential splicing. Most of the late gene products are viral structural proteins and other proteins involved in virion assembly, which include hexon, penton base, knobbed fiber, 100k protein, pIX, pIV and IVa2.

Adenoviruses enter cells by receptor-mediated endocytosis. Once the viral DNA is released into the nucleus, the viral early genes are transcribed, leading to DNA replication by E2 gene products. The viral DNA replication also initiates the late phase where gene expression by the MLP increases, which in turn results in a high production of all the

structural proteins that assemble together with viral genome in the nucleus. The newly synthesized virions are released from the cell by induction of cell lysis.

1.1.4.4 Adenoviral vector development and production

Most of the early generation adenoviral vectors are derived from Ad serotype 5. The replication-defective adenovirus (Ad) vector can be generated by replacing viral sequences such as E1, E2, E3 or E4 in viral DNA by the foreign cDNA. As described above, the adenoviral E1A genes are necessary for the activation of most of the viral promoters and the expression of both early and late genes. Thus, the removal of the E1 coding region results in replication defective virus. The first generation adenoviral vectors were specifically designed to replace their E1 region with the sequence of the gene of interest, so

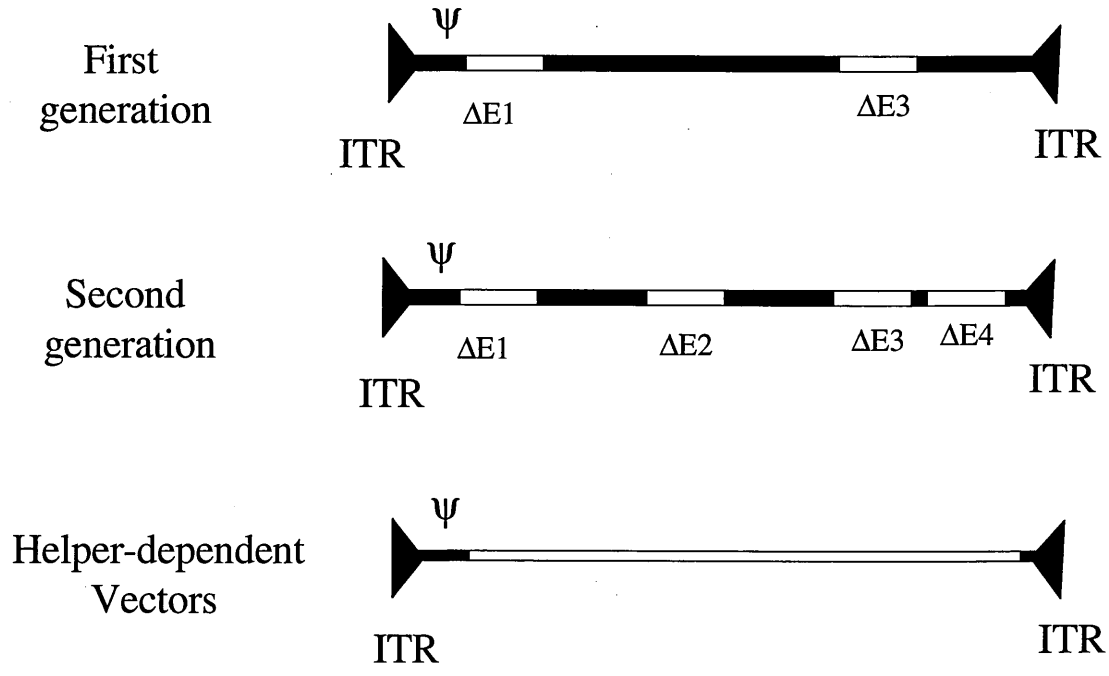


Figure 1.4 Genomic structure of first-generation, second-generation and helper-dependent vectors. Regions that have deleted are indicated by open boxes. Adapted from Mcconnell (2004).

that the recombinant virus could not replicate but was able to express the inserted transgene upon infection. The ability to generate E1 deleted vectors is made possible by the existence of cell lines that provide the E1 gene products *in trans*. One of the most frequently used cell line for this purpose is the 293A cell line, which is a human embryonic kidney-derived line that is immortalized by sheared fragments of adenovirus type 5 DNA (Graham et al., 1977). Production of E1-deleated vectors is usually carried out by homologous recombination or site directed recombination in the mammalian cells between constructs carrying the left and right end of the adenoviral genome. The cloning capacity of the first generation Ad vectors can be further increased by deletion of additional nonessential genes such as the E3 region. Combining the E1 and E3 deletions allows approximately 8.3 kb for insertion of therapeutic genes in one recombinant adenovirus. However, recent data has suggested that the expression of E3 genes from the recombinant vector may be beneficial *in vivo* because of its ability to dampen the host immune responses against the viral vector (Bruder et al., 1997).

Although first-generation vectors have been proven to be highly efficient as vehicles for gene delivery, one of the major challenges for using these vectors at a clinical level is the host immune response against the therapeutic vector. To overcome this problem, vectors deleted for multiple genes have been created to inhibit viral gene expression more effectively. These vectors, known as second-generation adenoviral vectors (**Figure 1.4**), are usually deleted in E2 and E4 coding sequences to reduce the host immune response against the adenovector and also have the benefit of a large capacity for transgene insertion due to the deletion. Experiments in immune-competent mice demonstrate that the transgene expression from the second-generation adenovectors was sustained much longer than the

first-generation vectors (Amalfitano et al., 1998). But the most promising approach for long-term gene expression with the adenoviral vectors is that of gutted, or helper-dependent adenovirus vectors (Alba et al., 2005), where all of the viral structural genes are deleted from the viral chromosome, leaving just the two ITRs and the packing signals. Such vectors can accommodate up to 37 kb of transgene sequences. The presence of a helper virus that provides the functions that required for replication and assembly of the helper-dependent adenovector. The main problem to date is the inability to completely separate virions containing the helper-dependent chromosome from those containing the helper virus genome (Sandig et al., 2000). However, *in vivo* studies using helper-dependent vectors have produced some promising results (Ehrhardt & Kay, 2002).

1.1.5 Gene therapy application:

1.1.5.1 Gene therapy for genetic diseases

Gene therapy is very attractive particularly for diseases that currently do not have any effective treatment options, and it is probably easier for targeting monogenic disorders than for complex diseases with multiple defective genes. The most obvious application for gene therapy is the replacement of a defective gene with its functional counterpart in order to restore normal physiological function and thereby reverse the diseases state. A successful gene therapy approach for monogenic diseases requires a stable transfer of the gene into target cells to insure the permanent correction of the disorder. There are two types of gene therapy approaches for inherited genetic disorders. The first approach is germ cell gene therapy, where the corrective gene is inserted into a sperm cell or ova and will therefore be incorporated into each cell of the new individual thus upon. However, genetic modification of the human germline is not allowed in any country yet. The second method is somatic

gene therapy. This approach involves a corrective gene transfer into somatic cells of an individual in order to restore the normal gene function. So far, about 90 trials for inherited monogenic disorders have been reported in the literature (Edelstein et al., 2004). Here, I briefly summarize the current advances and the future direction of gene therapy for different inherited monogenic diseases.

1.1.5.1.1 Cystic Fibrosis

Cystic fibrosis (CF) is caused by mutations in a gene named cystic fibrosis transmembrane regulator (CFTR), which is located on chromosome 7 and acts as a membrane chloride channel (Drumm et al., 1991). Mutations in the CFTR gene result in chronic lung infection, pancreatic dysfunction and diabetes mellitus. It is the most common autosomal recessive disorder in Caucasians, which affects about 1 in 2500 live births. In theory, CF states can be improved by restoring the mutant CFTR gene function, which can be achieved by transferring a wild type CFTR gene in the target cells with the gene therapy vector. In the early days, adenovirus-based vectors were widely used in most of the CF gene therapy experiments. But, it has become clear that these vectors are not best suited for CF gene therapy due to their poor gene transfer ability into the airway epithelial cells and also due to the host immune response against the adenovirus-based vectors (Crystal et al., 1994). In contrast, AAV-based vectors become more attractive for CF gene therapy because of their safety profile, broad tissue tropism, long-term gene expression and their ability to escape the host immune surveillance. Early trials for CF gene therapy using AAV did not induce inflammation, but showed inadequate amount of CFTR-gene transfer (Flotte et al., 1996). Target Genetics Corporation carried out several clinical trials with an AAV-

based vector tgAAVCF, which contains the complete human CFTR cDNA and uses AAV ITR-promoter elements to express the therapeutic gene. They administered this vector in an aerosolized form and were able to show that a single administration of the virus was well tolerated and safe, but no therapeutic benefits were observed in any patients due to lack of CFTR gene expression (Aitken et al., 2001). A recently reported phase I clinical trial using second-generation AAV2 vector expressing CFTR gene also showed the limited transduction of the therapeutic gene into the airway epithelial cells. The low transduction efficiency is thought to result in the increased inflammation and sputum barrier in patients with moderate to severe disease. Several nanoparticle formulations have been developed to increase the transduction efficiency for the CFTR gene into the target cells (Zabner et al., 1997). But no treatment efficacy has been noted. For a successful CF gene therapy, we have to overcome the problems associated with gene transfer to airway epithelial cells.

1.1.5.1.2 Hemophilia

Hemophilia A and B are inherited bleeding disorders that affect one out of 34,500 men (Bell et al., 1995). This disease is caused by a mutation in the Factor VIII and IX gene respectively, which are involved in blood clotting. Several characteristics make hemophilia an ideal candidate for a gene therapeutic approach: i) only a relatively small amount of proteins is required for phenotypic correction, ii) the therapeutic gene is small (Factor IX cDNA 1.4kb long) and it can easily be packaged into different viral vectors, and iii) because the Factor IX protein is secreted into the blood stream, one can target different organs such as muscle or liver to express the therapeutic gene. Most of the earlier attempts for gene therapy for hemophilia were based on adenoviral vectors. Adenovectors were initially attractive for hemophilia gene therapy because of their natural ability to transduce

hepatocytes. Although, preclinical studies with the first generation replication incompetent adenoviral vectors in both murine and canine hemophilia models showed promising results, the host immune response against these vectors compromised their safety and also inhibited long term therapeutic gene expression. The third generation gutless adenovector carrying Factor VIII gene for hemophilia A has shown a significant reduction in the immune response and hepatotoxicity (Ehrhardt et al., 2003), but its long term therapeutic gene expression is compromised by the formation of neutralizing antibodies against the therapeutic Factor VIII protein. The host immune response against the AAV-based vectors is much lower than the adenovectors and produces some very promising preclinical results for hemophilia gene therapy. So far, two clinical trials have been reported using AAV2 vector to deliver Factor IX to the muscle (Manno et al., 2003) and to the liver, via hepatic artery infusion (Kaiser, 2004). In the first clinical trial with adenovirus, muscle delivery increased the Factor IX expression only about 1%, whereas a 12% increase was detected in one of the patient treated by hepatic artery delivery of the rAAV vector. However, this increased Factor IX expression was followed by a transient elevation in serum transaminase levels and a loss of the therapeutic gene expression; it was speculated that the host immune response against the AAV vector lead to the destruction of the transduced cells (Sabatino et al., 2005). The main challenge for hemophilia gene therapy is how to avoid the induction of the host immune responses after the delivery of the therapeutic gene. Reduction of the therapeutic gene expression is often attributed to cell–mediated and humoral immune responses against the transduced target cells and transgene produced. Viral vectors used to deliver the therapeutic gene act as an immunological adjuvant and

amplify this response. Several strategies have been investigated for inducing immune tolerance to the Factor VIII and IX transgene products (Manno et al., 2006).

1.1.5.1.3 Muscular Dystrophy

Muscular Dystrophy (MD) is caused by a large deletion of the dystrophin gene that leads to a destabilization and subsequent degeneration of muscle tissue (Culligan et al., 1998). MD is a good candidate for gene therapy because the successful transfer of the wild type dystrophin gene to the muscle tissue should lead to the correction of the disease's state. But, the dystrophin gene is a very large gene that encodes a 3685 amino acid protein and 14 kb cDNA (Nobile et al., 1997). This is a significant challenge for MD gene therapy, because only a few viral vectors are currently available that will be able to package such a large transgene as dystrophin. A phase I trial has been initiated using a plasmid dystrophin DNA directly injected into the muscle to determine the tolerability and safety as well as gene expression (Thioudellet et al., 2002). In this trial, a low level of dystrophin expression was detected for up to 3 weeks in the muscle fibers of six out of nine patients (Romero et al., 2004). Gutless adenoviral vectors have been used to deliver dystrophin genes into the muscle of the mdx transgenic model of MD. But the success rate was limited by the host immune response against the viral vectors and inefficient infection of mature muscle tissues by this vector (Dudley et al., 2004). AAV vector system is very attractive because of its tropism to infect muscle cells and it's ability to escape immunological response *in vivo*. Unfortunately AAV vectors have a cloning capacity of only about 4 kb and cannot package full-length dystrophin cDNA. However, recent advances in creating mini- and microdystrophin genes have resulted in dystrophin expressing cassettes that can be packaged in the AAV vector (Gregorevic et al., 2006). But, it is yet to be determined if

these minimal dystrophin proteins can fully compensate for the lack of full-length dystrophin.

1.1.5.1.4 Severe combined Immunodeficiency

Severe Combined Immunodeficiency (SCID) is a group of rare monogenic disorders, which is commonly characterized by a block in T cell differentiation and a direct and indirect impairment of B cell immunity. Mutations in ten different genes have been found to cause ten distinct SCID phenotypes (Fisher et al., 2005). Recent understanding of the molecular basis of different SCID conditions has opened the door toward an alternative gene therapy, which is allogeinc hematopoietic stem cell transplantation.

The adenosine-deaminase (ADA) deficiency was the first inherited SCID disease to be treated with a gene therapy approach (Culver et al., 1991). This fatal inherited disorder is characterized by a defect in purine metabolism pathway that leads to impaired immune functions, recurrent infections and systemic metabolic abnormalities. A strong rationale for somatic gene therapy and the need for alternative therapy led to the design of the earliest human gene therapy clinical trial, which was based on retroviral-mediated gene transfer of the normal ADA gene into autologus hematopoietic stem cells (HSCs) or peripheral blood lymphocytes (PBLs). Most of these early gene therapy trials, which mainly used retroviral vector to transfer ADA gene into HSCs and PBLs, have proven to be safe and feasible (Bordignon & al., 1995). However, all patients in these trials received conventional enzyme-replacement therapy, which abolished the growth advantage for gene corrected cells and interfered with the proper evaluation of the gene transfer efficacy. It is only recently that the clinical efficacy of the ADA gene therapy has been examined in the absence of enzyme replacement therapy (Aiuti et al., 2002a). Results from these trials

showed that engineered PBLs were able to restore the T-cell defect, but were insufficient for systemic correction and detoxification. However, gene transfer in bone marrow stem cells showed a full immunological and metabolic correction of the ADA defect (Aiuti et al., 2002b).

In 2000, Cavazzana-Calvo and colleagues from the Necker Hospital in Paris (Cavazzana-Calvo et al., 2000) used a gene therapy approach to treat ten infants that were born with X-linked SCID. This type of SCID is the most common of all SCID syndromes, which is caused by mutations in the γ_C subunit of the interleukin 2 cytokines receptor family that leads to impairment of T, B and NK-cell development. In their gene therapy protocols, the authors used cytokines-stimulated, bone marrow-derived, autologous $CD34^+$ cells that were repeatedly transduced with an oncoretroviral vector carrying the normal γ_C gene. Approximately 15-20 X 10^6 engineered $CD34^+$ cells were infused back to the patient without any conditioning. Significant immune reconstitution occurred in all except one infant. T-cell levels increased from near undetectable levels to normal limits within 3 months of infusion and these modified T-cells were able to induce an appropriate immune response against a variety of antigens. The therapeutic transgene was detectable in almost 100% of circulating T cells. Unfortunately, almost 5 years after therapy, three patients that were involved in this trial developed T-cell leukaemia, both associated with an insertional mutagenesis event (Hacein-Bey-Abina et al., 2003, Marshall el al., 2003, Check et al., 2005). The activation of the LMO2 proto-oncogene at the site of vector integration played a key role in the development of leukaemia (Hacein-Bey-Abina et al., 2003), but other factors may have contributed as well including the effect of γ_C transgene (Dave et al., 2004) or abnormal proliferative advantage of corrected cells. This serious adverse side effect in a

trial that initially produced such promising results has slowed down the expansion of human gene therapy.

1.1.6 Gene therapy in neoplastic Diseases

Cancer is a multi-stage genetic disease that involves alteration in the multiple molecular pathways related to growth control and apoptosis in order to support uncontrolled growth of the cancer cells and support their ability to invade and metastasize (Hanahan & Weinberg, 2000). The interaction of cancer cells with their microenvironment, including stroma, extrcellular matrix, immune system, and cells necessary for induction of angiogenesis to support tumor growth is critical for tumorogenesis. Thus, there are many genes that have been identified in recent years, which can be a potential target for novel cancer therapy. Catalyzed by the finding of these new targets for cancer therapy, there has been a dramatic increase in developing gene therapy approaches for the treatment of cancer. The new knowledge of the molecular mechanisms underlying oncogenesis and the development of viral vector as a vehicle for gene delivery has permitted the formulation of the concept of cancer gene therapy. In this part of the chapter, I will discuss various strategies of cancer gene therapy.

1.1.6.1 Strategies for cancer gene therapy

Strategies for cancer gene therapy adopt ideas and technologies ranging from generating the immune response against tumor antigens to directly attacking on tumor cells. All the new understandings about the mechanisms of molecular alteration in tumorgenesis have contributed a great deal to the various approaches to cancer gene therapies. Some of the most popular approaches that have been explored to date are described below.

1.1.6.1.1 Transfer of tumor suppressor gene

The loss of tumor suppressor gene functions is one of the key characteristics in many human malignancies. In the most direct "gene replacements" approach of cancer gene therapy, tumor-suppressor genes are delivered and expressed in cancer cells in which these genes are defective, resulting in cell death and growth arrest. Several tumor suppressor genes have been isolated in recent years. Examples of tumor suppressor genes are –the retinoblastoma pRB, APC, PTEN and the p53. The expression of each of these genes in tumor cells *in vitro* causes an acute change in cell physiology and gene expression, resulting in cell cycle arrest or death (McCormick, 2001). Vectors expressing tumor suppressor genes such as p53 injected directly into the tumor show some promise both *in vivo* and clinically (Roth et al., 1999). But one of the major problems of the gene replacement approach of cancer gene therapy is that almost every cell in the tumor would need to be infected with vector carrying tumor-suppressor gene in order to achieve clinically relevant therapeutic efficacy, which is an enormous technical hurdle with the current vector technology in hand.

1.1.6.1.2 Suicide genes-enzyme/pro-drug approach

One of the most popular ways to achieve direct targeting of cancer cells with gene therapy is the delivery of suicide genes to cancer cells. In this approach, vectors expressing suicide genes allow the cancer cells to metabolize a harmless prodrug, administered separately, into a potent cytotoxic that does not only kill the transduced cell with the suicide gene, but also can diffuse into the neighboring cells and create a bystander effect. Several prodrug-enzyme combinations are evaluated for this approach, but the herpes simplex virus thymidine kinase (HSV-tk) has been the most popular and most extensively

used in the clinic. The HSV-tk gene converts inactive prodrug ganciclovir to the phosphorylated active form, which becomes incorporated into DNA during cell division, thereby blocking DNA synthesis. The vector carrying suicide genes can be injected directly into the tumor mass or delivered systemically and targeted to tumor cells by using the tumor-specific expression elements. Clinical studies with suicide gene therapy have proven to be safe, but not sufficient enough to show clinically relevant therapeutic efficacy. A Phase III clinical study of retrovirus- encoded HSV-tk showed no patients benefit (Roth et al., 1996) Adenovirus expressing HSV-tk injected directly into brain looked slightly more promising, with some survival benefit in a small number of patients (Sandmair et al., 1999). However, suicide gene activity needs to be enhanced to improve its efficacy.

1.1.6.1.3 Anti-angiogeneic therapy

In the last twenty years, remarkable progress has been made in understanding the molecular mechanisms of angiogenesis. Molecules such as vascular endothelial growth factors (VEGF) or TIMP-2 have been identified as a culprit that supports angiogenesis in the tumor microenvironment. These molecules are excellent candidates for cancer gene therapy because the inhibition of their activity can lead to significant tumor growth suppression and result in bystander effects. Angiogenesis can also be inhibited by a constitutive expression of anti-angiogeneic molecules such as angiostatin or endostatin. Adenovirus vector expressing a soluble form of VEGF receptor were recently shown to inhibit tumor growth in mouse models (Ogawa et al., 2002). An adenovirus expressing secretable endostatin was able to inhibit tumor growth *in vivo* (Li et al., 2006). Vectors have been designed to express siRNA to target VEGF and VEGF receptors. But these approaches so far are not very successful in the clinic because a relatively long-term

expression of the therapeutic gene, at least until regression or apoptosis of tumor cells are deprived of their nutrition.

1.1.6.1.4 Immunomodulatory approaches

Recent research has identified numerous mechanisms used by cancer cells to escape host immune surveillance. These include expressing high amounts of immunosuppressive cytokines like interleukin (IL)-10 or TGF-β, lowering the expression of MHC class I and II protein, impairing growth and differentiation of effectors immune cells that down regulate expression of different costimulatory molecules (Rosenberg, 1992). Thus, using viral vectors to express some of these immunostimualtory genes to activate the host immune system is an attractive strategy. The types of immunostimulatory genes used in the clinical and pre-clinical settings in cancer gene therapy are IL-2. IL-4, IL-12, IL-6 and tumor necrosis factor, interferon-γ and T-cell costimulatory molecules such as B7.1 and B7.2. The viral vectors carrying these molecules are also being used ex-vivo to develop cell-based vaccines, in which case the patient can be vaccinated with autologous tumor cells infected with gene therapy vector. If successful, immunomodulation approaches have the potential to develop as systemic cancer therapies.

1.1.6.1.5 Fusogenic membrane glycoprotein

Fusogenic membrane glycoprotein (FMG) is derived from viral genes, which mediate viral binding and subsequent internalization via viral envelope fused with cell membrane. In vitro, when FMG is expressed in cell monolayer containing the appropriate receptor, massive cell-cell fusion occurs and multi-nuclei syncitia are formed. All enveloped viruses enter cells by protein mediated membrane fusion. These include viruses from diverse groups such as retroviruses, paramyxoviruses and orthomyxoviruses. These viruses enter

cells primarily by one of two pathways: entry following direct fusion between the viral and cell membranes at the cell surface or entry by endocytosis and fusion between the viral and endosomal membrane. The first mechanisms is pH independent and unlike the second one. But both routs of entry require initial binding via the receptor-binding domain to a cellular receptor. The N-terminus of each fusion contains a hydrophobic region, the 'fusion peptide', which in the stable conformation inserts into the target membrane during membrane fusion.

The idea of using FMG as a therapeutic gene against cancer relies on the induction of extensive cell-cell fusion of tumor cells to generate nonviable syncitia, resulting in a desire cytoreductive effect (Bateman et al., 2002). For this to occur, it is more likely to be effective using FMG that undergo pH independent fusion. The FMG used in the future studies in this thesis is the envelope of a C-type retrovirus, Gibbon ape leukemia virus (GALV), which will be discussed in detail (Hanger et al., 2000).

The GALV envelope shows very close homology to other C- type retrovirus envelopes such as Murine leukaemia virus and Feline leukemia virus. This envelope is composed of two polypeptides formed following the cleavage of the 85 kDa precursor protein. The large 70 kDa surface polypeptides are completely extracellular and correspond to the N-terminal region of the precursor. The smaller 15 kDa transmembrane polypeptide corresponds to the C- terminal region of the precursor. The receptor for GALV is a sodium-dependent phosphate symporter, PiT 1 (Takeuchi et al., 1992) and is widely expressed in many tissues.

1.1.7 Oncolytic virus

The therapeutic efficacy of most of the cancer gene therapy approaches is significantly compromised by the inability of the current viral vectors to deliver gene efficiently *in vivo*. To overcome this problem, people have used the viruses' ability to spread from their site of infection and infect the neighboring cells. As a consequence of virus infection, infected cells are killed as they become the factories for producing the new infectious viral particles (**Figure 1.5**). This approach not only amplifies the application therapeutic genes in a tumor selective manner, but also uses its ability to lyses and kills the infected cell and eliminates the tumor cells selectively. The success of this approach depends on our ability to engineer or select viruses that replicate specifically in tumor cells, but not in the normal cells.

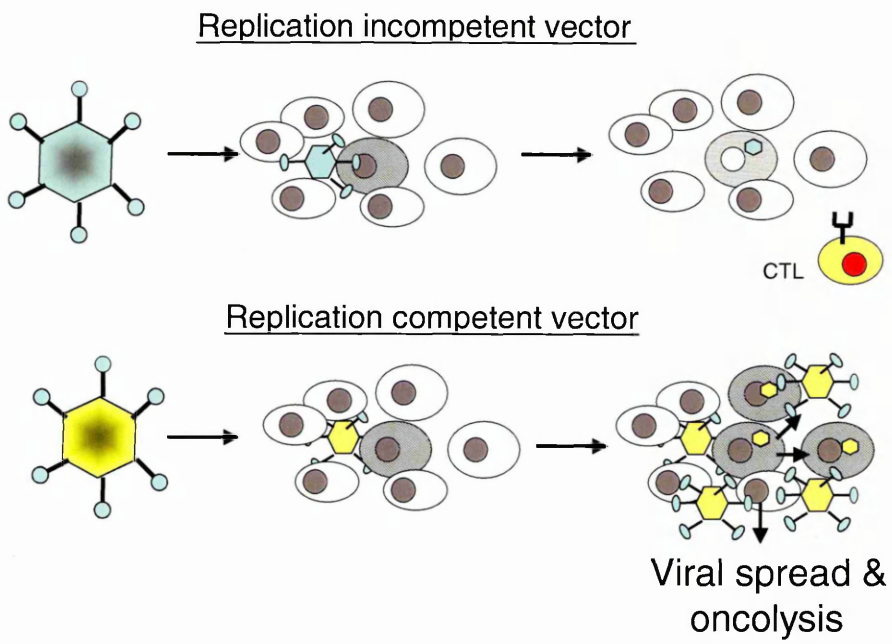


Figure 1.5 Replication-defective verses replication-competent vector for cancer gene therapy. Replication-defective vectors are limited transduction efficiency, oncolytic viruses are capable of progressive replicative spread, resulting in an amplification of the initial input dose and more effective in eliminating tumor. Adapted from Yoon (2001).

These viruses, termed oncolytic viruses, are essentially tumor-specific, self-replicating, lysis-inducing cancer killers. In this part of the chapter, we will summarize what is currently known about many of these oncolytic viruses and discuss some of the obstacles of this approach of cancer gene therapy. Many oncolytic viruses belonging to several viral families have been identified or engineered. They include herpes simplex viruses, adenovirus, retroviruses paramyxoviruses and poxviruses. These viruses can be categorized into four major groups on the basis of their oncolytic restriction: I) mutation/deletion derived viruses, II) transcriptional targeted oncolytic viruses, III) transductional targeted oncolytic viruses and IV) 'naturally smart' viruses.

1.1.7.1 Mutation/deletion derived oncolytic viruses

One of the earliest approaches takes advantage of tumor specific changes that allow preferential replication of the virus only in target cancer cells by introducing strategic mutation or deletion in the viral genome, thus inhibiting natural viral replication in the nontargeted normal cells.

The tumor suppressor gene p53, so-called guardian of the genome, is responsible for inducing cell-growth arrest and apoptosis in response to cellular stress, DNA damage or viral infection. The wild-type adenovirus naturally suppresses p53 activity by expressing E1B, which prevents apoptosis and allows the virus replication to occur (Yew & Berk, 1992). Since almost 50% of all tumors have deletion or mutations in the p53 gene, E1B is not necessary for the virus to replicate in these cells and the virus should replicate efficiently. However, in normal, quiescent cells, E1B deleted viruses are unable to replicate

A. Tumor targeting strategies

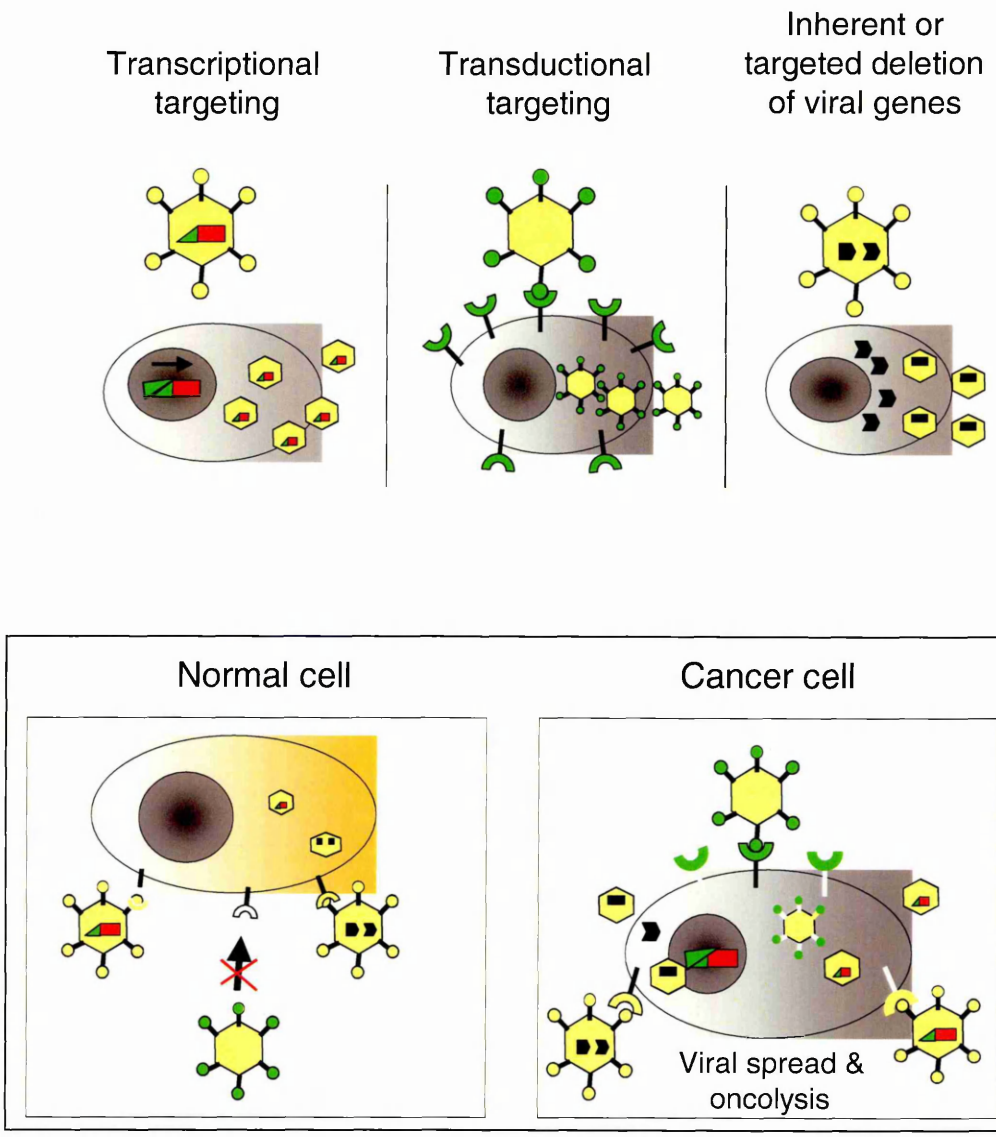
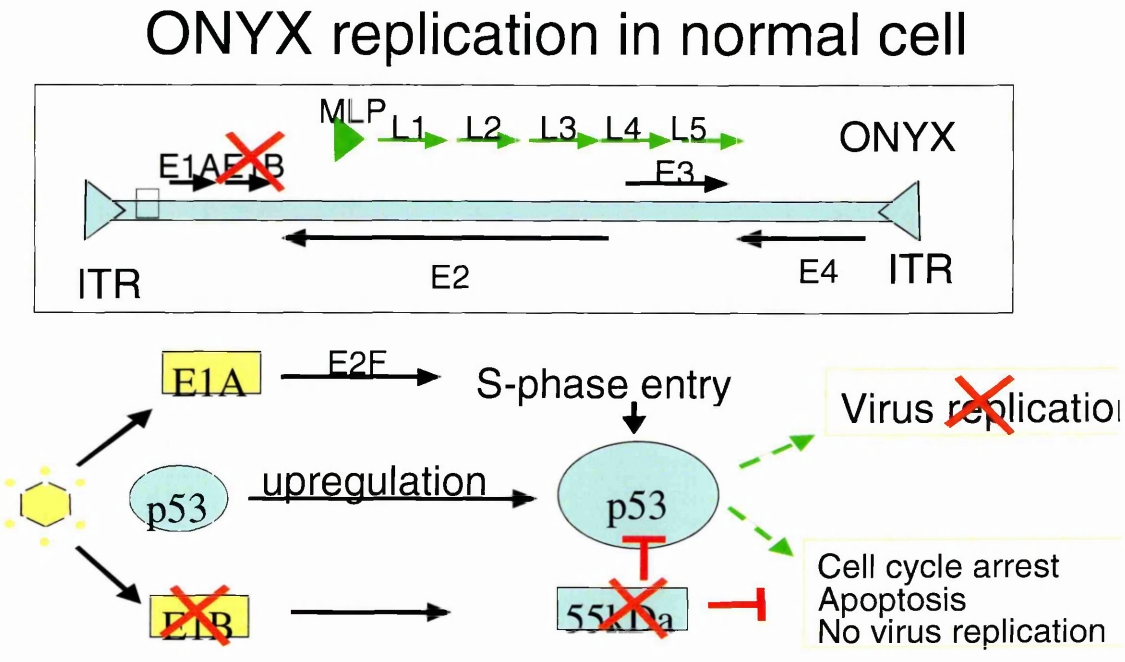


Figure 1.6 Retargeting of adenoviral vector for cancer gene therapy. Targeting can be achieve at three levels: (1) expression of adenoviral genes can be limited to target tissue by the introduction of tumor/tissue specific promoters (transcriptional); (2) adenovirus can be conjugated with retargeting moieties to avoid binding to its native receptor CAR and introducing binding to new receptors present on target cells (transductional); (3) replicative essential viral gene can be deleted selectively so the vector can not replicate in any cells except the target cells (targeted deletion).

because of their inability to inhibit wt p53 activity and prevent apoptosis in the infected cells – at least in theory (**Figure 1.7**). A virus – known as ONYX –015 or dl1520 (Bischoff et al., 1996) deleted for E1B gene is one of the first engineered oncolytic virus used in clinical trials. The ONYX-015 has been used successfully to treat different tumor models in animals, but it has been less successful in treating cancer in humans (Ries & Korn, 2002). ONYX-015 showed some therapeutic benefits when used in combination with standard chemotherapies (Heise et al., 1997). But several reports are showing that ONYX-015 can replicate in some tumor cell lines that retain wild-type p53 (Goodrum & Ornelles, 1998) (Turnell et al., 1999). These findings have raised concerns about safety and replication selectivity of ONYX-015 and the specificity of ONYX-015 for p53-deficient cells remains controversial (Dix et al., 2001). Many different gene deletion strategies have been used to develop the gene deleted viruses show, which some promising selectivity and killing activity *in vitro* culture systems, even against the tumor in animal models.

A.



B. ONYX replication in cancer cell

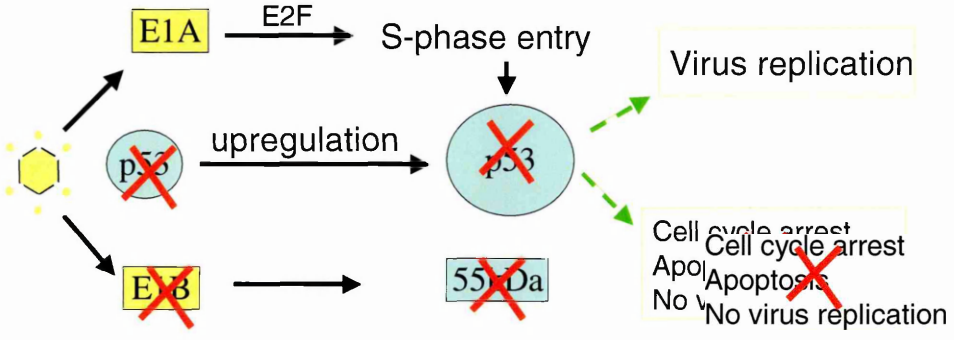


Figure 1.7 Interaction between adenovirus-encoded proteins and cellular factor that facilitate ONYX-015 replication and host cell disruption. A. Early gene E1A expression induces forced entry into S phase, resulting in activation of the p53 pathway leading to cell death. Adenovirus counteracts p53 by E1B-55kD and E4orf6 that bind to, and inactivate p53. ONYX-015 is E1B-55kD deleted, which cannot replicate in normal cells with wild type p53. But tumor cell with dysfunction p53 pathway able to support ONYX-105 replication because they cannot activate p53 dependent apoptotic pathway.

However, none of them stand out when they are used in clinical trials. Additional genetic modifications need to be developed for increasing the effectiveness of the mutation/deletion derived oncolytic viruses.

1.1.7.2 Transcriptionally targeted oncolytic viruses

Another approach to achieve tumor-selective replication involves expressing viral genes under promoters that are only functional in tumor cells. This strategy has been used primarily with the adenovirus and herpes simplex virus (HSV). Researchers have employed various tumor-specific promoters (hTERT, Try, CEA) or tissue-specific promoters (MUC1, PSA, AFP) to drive the replication-essential viral gene such as the adenoviral E1A in order

to achieve tumor selective replication. One such tumor-specific promoters, which shows a lot of promise *in vitro* and animal models is the promoter that derived the gene that encodes the telomerase reverse transcriptase (hTERT). The hTERT protein is necessary for the extension telomeres, and expresses at high levels in cells that are rapidly dividing. Over 85% of human tumors overexpress the hTERT gene (Irving et al., 2004). A number of studies have demonstrated that the oncolytic selectivity can be achieved by using the hTERT promoter to derive the expression of early gene, such as adenoviral E1 gene. Another tissue specific promoter MUC1 has been used to express E1A, supporting virus replication selectively in breast cancer cells in which MUC1 is aberrantly expressed (Chen et al., 2005). Using these tumor selective promoters to develop conditionally replicating viral vectors shows success in both the preclinical and the clinical settings, but the commercial and clinical development and use of these viral vectors for cancer gene therapy might be complicated by a dominating patent on the use of tissue-specific promoters for gene therapy (Novartis US patent 5,998,205, Hallenbeck, Chang and Chiang 1999).

1.1.7.3 Transductional targeted oncolytic viruses

Different viruses use different cellular receptors and distinct sequential steps to infect target cells. For examples, two different cellular receptors are responsible for adenovirus entry. First, the knob domain of the adenovirus fiber capsid protein binds to the cellular receptor CAR on the cell membrane of the target cells and then the RGD peptide sequences in the penton base interacts with the cellular integrin receptors to initiate internalization of the virion (Wickham et al., 1993). During tumorigenesis, various genetic and epigenetic events cause overexpression of so-called 'tumor antigens' on the surface of tumor cells. As recognition of specific cell surface receptors is the key initial step for virus entry and

productive infection, researchers are able to engineer viruses that only recognize and use the antigen present on the tumor cell surface to enter the target cells, which then restrict replication of an oncolytic virus to malignant cells. The first step of this targeting strategy requires creating a blind virus that no longer recognizes its normal cellular receptors for entry. This receptor blind virus then can be retargeted to tumor cells by using bifunctional crosslinkers or structural modification to recognize the antigens that are predominantly expressed on the tumor cell surface. The oncolytic measles virus has been successfully modified by this strategy. During normal infection, measles virus binds by its hameagglutinin (H) attachment protein to one of two cellular receptors: CD46 and SLAM (Dorig et al., 1993) (Tatsuo et al. 2000). By introducing mutations in SLAM and CD46 binding domains of the measles virus H-protein, Vongpunsawad et al created a blind virus, which was then subsequently engineered to express H-protein fused with growth factors such as EGF and IGF, as well as single-chain antibodies against tumor antigens such as CEA, CD20 or CD38 (Vongpunsawad et at., 2004, Nakamura et al., 2005).

1.1.7.4 'Naturally smart' viruses

Many viruses have a natural tropism for tumor cells for different reasons. For example, RNA viruses such as vesicular stomatitis virus (VSV) (Lichty et al., 2004), reovirus (Coffey et al., 1998) or Newcastle diseases virus (Cassel & Garrett, 1965) replicate selectively in tumor cells because these cells fail to mount a protective interferon response against the viral infection. These viruses demonstrated oncolytic potency in studies from tissue culture up to human clinical trials, but difficulties in engineering and uncleared restriction mechanisms somewhat hampered the progress to take these viruses into the clinic (Russell, 2002).

1.1.7.5 Conclusion and future direction

As vectors of cancer gene therapy, oncolytic viruses face similar challenges as any nonreplicating virus would. The major problem is that the host immune responses to circulating viral particles prevent vectors from having any prolonged persistence. To replicate, oncolytic viruses must produce viral protein intracellularly. These protein can also be presented on major histocompatibility complex class I (MHC I) protein as viral antigen, attract cytotoxic T lymphocytes (CTLs), which are responsible for clearing intracellular pathogens and will arbitrarily destroy infected cells that are attempting to produce therapeutic oncolytic viruses. Conversely, attracting CTLs at the site of infection in the tumor could be therapeutically beneficial to patients. The viral infection could produce wide variety of chemo- and cytokines, which can create a suitable environment for CTLs to eliminate cancer cells more efficiently and also help antigen-presenting cells to recognize tumor associated antigens that lead to generate immune response against the tumor and break the immune tolerance for tumor. Thus, the advantages and disadvantages of immune responses against the vectors in oncolytic virotherapy must be further examined for each vector used. Also, the preexisting humoral immunity against the viral vectors such as adenoviral vector is a major hurdle for systemic delivery of these vectors. To evade these antibodies against the therapeutic vectors, formulations based on cationic liposomes have been produced to encapsulate in a lipid bilayer and protecting the vector from initial detection and agglutination (Yotnda et al., 2002). Formulating adenovirus in collagen-based matrices (Siemens et al., 2001) and polyethylene glycol (PEG) has been shown to protect the viral vector from the host immune system (Croyle et al., 2002). In addition, different immunosuppressive drugs have been used to inhibit B-cell maturation and mast

cell activation (Bouvet et al., 1998). Viral immunomodulatory gene, such as adenoviral E3 gene that has been deleted from most of the adenoviral vectors, could be useful for controlling the host immune response against the therapeutic vectors (Wang et al., 2003b).

In the future, it is likely that a successful oncolytic virus will use a combination of different methods of selectivity to ensure the maximum safety and therapeutic efficacy. The oncolytic activity itself may not be sufficient enough to eradicate the tumor burden. To enhance the therapeutic potency at the level where clinically relevant therapeutic efficacy can be achieved by an oncolytic virus, the therapeutic vector may need to carry an additional therapeutic genes such as suicide gene HSV-TK or immunostimulatory gene such as TNF-α, IL-24/mda-7 (Lambright et al., 2001). Also, multiple genetic restriction points for enhanced tumor specificity and vector-specific immune suppressant to overcome the host immune response is the key to create a vector that will be safe and effective for cancer gene therapy in the clinic.

1.2 mRNA degradation and stability in regulation of gene expression.

1.2.1 Introduction

It is becoming increasingly clear that controlling the rates of mRNA decay is a key factor in the regulation of gene expression (Shim & Karin, 2002) (Wilusz & Wilusz, 2004). Controlling gene expression at mRNA level is faster and more economic than other regulatory mechanisms such as selective protein degradation in the proteasoms. The rate of mRNA decay varies considerably from one mRNA species to another and can be altered by many extracellular stimuli ranging from development, hormonal and nutritional, to environmental stresses

(Guhaniyogi & Brewer, 2001). Genes like most cytokines and many proto-oncogenes produce mRNAs that are unstable in quiescent environments, but are considerably stabilized upon appropriate stimulation. In this part of the chapter, I will provide a brief overview of all the important components involving the process of mRNA decay and stability.

1.2.2 mRNA decay pathway in mammalian cells

The regulation of mRNA decay in mammalian cells is a tightly controlled process, which is orchestrated by the interaction between the mRNA's structural components and the *trans*-acting factors. The mRNA components include the 5' untranslated region (UTR), the 5'-cap structure, the protein-coding region, 3'UTR and the 3' polyadenylation [poly(A)] tail. Experiments from both yeast and mammalian systems reveal that the majority of the physiological mRNA decay is initiated by a deadenylation-dependent pathway, which starts with the selective removal of the 3' poly(A) tail followed by the 3'→5' decay by the poly(A) specific exonucleases. The length of the poly(A) tail is very important for deciding the stability of any mRNA (Korner & Wahle, 1997). In the stable transcripts, the 3' poly(A) tails form a complex with a highly conserved 70-kDa poly(A)-binding protein (PABP) that stabilizes the mRNA by protecting 3' poly(A) tail from deadenylation (Bernstein et al., 1989). The exosome, a complex of 10 or more 3'→5' exonucleases plays a key role in the mRNA turnover. Also the PABP bound to poly(A) tail interacts with the translation initiation factor eIF4 family proteins, which are bound at the 5'-cap region of the mRNA (Wells et al., 1998) and circularize the mRNA molecule, thereby initiating the assembly of the translation initiation complex at the 5' cap and increase the translation rate. The 5'-cap structure can also be the target for an exonucclease-dependent decapping activity, which then initiates the 5'→3' decay of the mRNA. The cap-binding eIF4 family protein can inhibit the 5' decapping activity and the presence of the adenylate-uridylate

rich (AU-rich) elements (ARE) in the 3'UTR of the mRNA can stimulate the decapping activity (Gao et al., 2001). In mammalian cells, the exosome mediated 3'→5' mRNA decay is more active than the 5'→3' decapping pathway (Mukherjee et al., 2002).

1.2.3 Control of mRNA decay by *cis*-acting elements (ARE)

Many cytokines and proto-oncogenes respond to the extracellular stimuli by transiently stabilizing their transcripts and inducing protein expression. But in the resting environment, the rapid disappearance of these mRNA is mediated by specific *cis*-acting elements found either in the coding region or, more frequently, within the 3' UTR region of the RNA. The most well studied *cis*-acting element involved in controlling the half-life of many mRNAs is AU-rich element (ARE) found in the 3'UTRs of these unstable mRNA. The first ARE was identify from the human granulocyte/macrophage colony stimulating factor mRNA, which was 51-nucleotide long and able to destabilize a very stable β-globin mRNA by its incorporation into the 3' UTR region (Shaw & Kamen, 1986). Since then, many different AREs have been discovered and extensively examined. These AREs can be quite variable in length and sequence, but a common feature of carrying multiple copies of AU-rich elements characterizes most of them. These AREs are divided into three major subclasses: (i) Class I AREs contains 1-3 copies of AUUUA repeats within the AU-rich region and are present in the proto-oncogene (*c-fos, c-myc*) and cytokines coding genes (*IL-4, IL-6*), (ii) Class II AREs contain 5-8 copies of AUUUA repeats and are only found in the cytokines mRNAs (GM-CSF, TNF-α), (iii) Class III AREs lack AUUUA repeats, but contain a long U-rich region- such elements are found in the *c-jun* proto-oncogene mRNA (Peng et al., 1996). The AREs in mammalian cells are more conserved than the sequences in the open reading frames of the mRNAs: for examples, only 45% of sequence homology was found in the coding region between murine and human IL-3, but the AU-rich

region in the 3'UTR were highly conserved with 93% sequence homology (Dorssers et al., 1987). The ARE-directed mRNA turnover is extremely heterogeneous in the rate of decay as well as in the decay mechanisms (Wilusz et al., 2001). This heterogeneity presumably arises from the diverse RNA-protein and protein-protein interactions that regulate this process. Specific protein binding to ARE-elements in the 3'UTR may be either sequence specific or dependent on the secondary structure of the ARE-elements formed in the mRNA. The mammalian 70-kDa heat shock protein (Hsp) has been identified in a cellular complex containing an ARE-binding protein as well as being detected in direct contact with ARE sequences (Laroia et al., 1999). It has been hypothesized that Hsp 70 and other chaperones may be recruited for unwinding the complex secondary structure of AREs to expose critical *cis*-acting sequences, which may then facilitate binding of all the necessary *trans*-acting factors involved in the mRNA turnover. The AREs activity can also be differentially regulated in response to specific extracellular stimuli and is cell lines dependent (Nair et al., 1994). *In vitro,* the ARE sequences are able to stimulate decapping {Gao, 2001 #131}, exosome recruitment {Mukherjee, 2002 #132} and poly(A) shortening. The ARE mediated mRNA destabilization involves AU-binding proteins (AUBPs) that physically recruit the exosome on the RNA to be degraded - as opposed to AUBPs involving in stabilizing ARE containing mRNAs are not able to interact with the exosome complex, thus protecting the transcript degradation (van Hoof & Parker, 2002). The ability of specific AREs to stabilize or destabilize a target mRNA is governed by the synchronized interaction between the *trans*-acting RNA-binding proteins, extracellular stimuli and intercellular signaling pathways.

1.2.4 *Trans*-acting factors regulating mRNA stability

Several AU-binding proteins have been identified, characterized and cloned over the past decade (Wilson et al., 1999). The binding of these proteins to mRNA containing an ARE can have either negative or positive effects on the turnover, translation and localization of the mRNA. Here we discuss a few well-characterized AUBPs known to play a significant role in influencing the stability of the ARE bearing mRNA.

1.2.4.1 Tristetraprolin

Tristetraprolin (TTP) belongs to a group of zinc finger proteins that is characterized by two copies of CCCH zinc finger motif (Thompson et al., 1996). TTP inhibits the TNF-α production from macrophages by directly binding to the ARE and destabilizing the TNF-α mRNA (Carballo et al., 1998) (Lai & Blackshear, 2001). The macrophages from the TTP deficient mice are inefficient in regulating the TNF-α mRNA stability, which leads to aberrant expression of TNF-α and develop a complex phenotype consisting of dermatitis, cachexia, myeloid hyperplasia and autoimmunity (Taylor et al., 1996). TTP serves as an adaptor protein that bridges the ARE-mRNA to exosome and initiates the mRNA degradation (Chen et al., 2001). This protein can exist in numerous phosphorylated forms and be affected by the signaling pathways such as mitogen-activated protein kinase (MAPK) or the p38 MAPK (Mahtani et al., 2001). The phosphorylation statuses of TTP also determine its ability to decay the ARE-mRNA.

1.2.4.2 AUF1

AUF1 is the most extensively characterized AUBP. It was first identified from an enriched cytosolic protein fraction, which is able to accelerate ARE-dependent c-myc mRNA decay *in vitro* (Zhang et al., 1993). AUF1 is expressed as four related isoforms (p37, p40, p42 and p45) generated by alternative splicing (Wagner et al., 1998). Different isoforms appear to display

different effects on mRNA turnover. AUF1 induced mRNA-decay is initiated by AUF1 forming multi-subunit complexes on ARE with itself as homodimers or heterodimers and with additional cellular factors such as eIF4G, PABP and the hsp70 (Laroia et al., 1999). Although the destabilizing function of AUF1 is well-documented, recent reports suggest that AUF1 may also be involved in the interaction with mRNA stabilizing factors, such as embryonic lethal abnormal vision (ELAV) (Levine et al., 1993) and HuR (Ma et al., 1996) and initiate stabilization of ARE bearing mRNAs (Myer et al., 1997). In vitro, the stabilizing effect of AUF1 is thought to be highly selective for class II AREs. The AUF1 expression is increased during T-cell activation where AUF1 selectively stabilizes the class II ARE containing cytokines mRNAs (Ming et al., 2001).

1.2.4.3 HuR

HuR belongs to the ELAV family protein that is known to bind the ARE motifs with three RNA recognition motif (RRM)-type RNA-binding domains; also it stabilizes and/or activates the translation of the target mRNA. This protein is ubiquitously expressed in all cell types (Antic & Keene, 1997) and has a high binding affinity for several AREs (Levy et al., 1998). By using the immunoprecipitation of the HuR containing RNA/protein complex to identify the global target mRNA for HuR, Gorospe's lab was able to show that 15% of 9600 analyzed genes are able to form a complex with HuR (Lopez de Silanes et al., 2004). The overexpression of HuR protein leads to the accumulation of ARE bearing target mRNAs. Although predominately nuclear (>90%), it shuttles between nucleus and cytoplasm, influencing mRNA translation and stability (Fan & Steitz, 1998); so moreover the subcellular localization is closely linked to its functions. The nucleocytoplasmic trafficking of HuR is modulated by HuR-binding proteins that interact with the nuclear

export factor CRM1 and initiates shuttling to cytoplasm (Gallouzi et al., 2001). The cytoplasmic localization of HuR is also known to be influenced by the activity of AMP-activated kinase (AMPK), an enzyme that has a key physiological role in the cellular response to stress (Hardie & Hawley, 2001). HuR is shown to interact with the endonucleolytic site in the target mRNA and to protect this site from endonuclease cleavage (Zhao et al., 2000).

1.2.4.4 *Hsp70*

During different cellular stress, mammalian cells respond by elevating the synthesis of a highly conserved set of proteins, known as heat shock proteins (Hsp). The primary function of the four major groups of Hsps (Hsp27, Hsp60, Hap70 and Hsp90) is to help proper protein folding *in vivo.* But recently, this family of proteins and more specially Hsp70, is implicated in playing an important role in the cytoplamic metabolism of lymphokine and other short-lived mRNAs by physiologically regulating the interaction between the AU-binding protein, AUF1 and the ARE-mRNAs. It also directly competes with other AUBPs during the heat shock, thus influencing the ability of these factors to stabilize or destabilize the ARE-mRNAs. It is thought that the competitive equilibrium between AUF1 and Hsp70 for ARE substrates is rapidly shifting in favor of Hsp70 under heat shock conditions, which contributes to the stabilization of these mRNAs at the elevated temperatures. Alternatively, the chaperone activity of Hsp70 alters the local RNA structures and influences the binding of the AUBPs to the ARE bearing mRNAs (Wilson et al., 2001).

1.2.5 Signal transduction pathways in regulation of mRNA stability

The ARE-mediated RNA decay is highly dependent on the stimuli induced signal transduction pathways. The ability of these signaling pathways to post-translationally modify

AUBPs by phosphorylation and alter the binding affinity towards ARE-mRNAs has a profound effect on the stability of many ARE-bearing mRNAs. Early reports describing the signaling pathway altering ARE-mRNAs included phorbol ester, antibodies induced T cell activation and TNF-α. In this section I will describe some examples of this regulation.

One of the most well studied signaling pathways that alter the stability of many ARE-bearing mRNAs is the p38-signaling cascade. p38 belongs to the large family of Mitogen-Activated Protein Kinases (MAPK). Different stresses, such as heat shock or UV rays activate this signaling cascade by dual tyrosine/threonine phosphorylation of p38, which then activates the downstream effectors and alter the interaction between AU-binding proteins and ARE-mRNAs. As described before, TTP directly interacts with the ARE of the TNF-α mRNA and induces decay. During arsenite stress, p38-MAPK mediates TTP phosphorylation, which leads to its association with 14-3-3 proteins and result in exclusion from stress granules (Stoecklin et al., 2004). Under these conditions, another AUBP HuR binds to the ARE of the TNF-α mRNAs in the stress granules and stabilizes the transcripts. But under mitochondrial stress, TTP remains unphosphorylated and can be localized to the stress granules where it binds to AREs and acts as a potent destabilizing factor (Stoecklin et al., 2004).

The AREs of other cytokines such as GM-CSF or IL-3 are able to regulate mRNA stability in response to calcium signaling {Bickel, 1992 #159}. During the T-cell activation, the stabilization of the IL-2 mRNA is mediated via two different cis-acting elements and signaling pathways (Chen et al., 2000). One of the pathways involves JNK signaling cascade activation and binding of two proteins, YB-1 and nucleolin to the 5'UTR of the IL-2 mRNA. The second pathway requires activation of protein kinase C (PKC) and/or calcineurin

signaling pathway and also binding an AUBP known as NF90 to the ARE at the 3'UTR to stabilize the IL-2 mRNA.

Many different environmental stresses induce signaling pathways also known to alter the rate of ARE-mRNAs decay. Among them, hypoxia is one of the well-characterized stresses that is known to induce VEGF through mRNA stabilization. A recent study demonstrated that the hypoxia-induced activation of stress signaling pathways like JNK and p38 is required for the selective VEGF mRNA stabilization (Pages et al., 2000). When exposed to UV light (UVC), the AU-binding protein HuR-dependent p21 mRNA stability was shown to be associated with the elevated retention of the HuR protein in the cytoplasms (Wang et al., 2000b).

The stimuli induce a signaling pathway and also change the ARE-mRNA turnover by post-translational modulating the activity of specific endonucleases. One such example is endonuclease G3BP, which interacts with the oncogene RAS. G3BP is a 52kDa endoribinuclease known to require site-specific phosphorylation for its catalytic activity (Gallouzi et al., 1998). The c-myc mRNA, which contains a high-affinity G3BP binding site at the 3'UTR region, can be cleaved by G3BP in a phosphorylation-dependent manner. In the serum stimulated cells, the G3BP gets phosphorylated by RAS and localizes to the nucleolus where G3BP cleaves the 3'UTR and destabilizes the c-myc mRNA (Tourriere et al., 2001).

1.2.6 Physiological significance

The ARE-dependent mRNA degradation functions as a sensor for regulating the adaptive cellular response to different physiological stimuli. Many biological processes such as development, aging, host defense, cell cycle regulation, apoptosis and angiogenesis require transient response in the gene expression; moreover the ARE-mediated control of mRNA stability is proven to be one of the most economic and efficient way of regulating gene

expression. The following are few examples of physiological conditions where ARE-mediated mRNA stability is proven to be extremely important.

1.2.6.1 Regulated mRNA stability

Throughout the development specific and timely gene expression is very critical. This regulation of gene expression is coordinated at multiple levels including transcriptional, post-transcriptional, mRNA and protein turnover. The aim of this section is to introduce few examples that illustrate the developmental role of mRNA turnover in the gene regulation.

1.2.6.1.1 ζ-to-α Hemoglobin gene switching

Hemoglobin exists as a tetramer, which is composed of two α chains and two β chains, encoded by α-globin and β-globin gene clusters. In humans, the α-chains consist of α and ζ forms, which express simultaneously during the embryonic yolk sac development. But a switch to exclusive production of α-chains occurs in enucleated erythroid cells of the fetal liver at 6-7 weeks of gestation (Russell et al., 1997). The exclusive production of the α-chains then persists throughout adulthood in the bone marrow. The total shutdown of the ζ-globin gene and exclusive synthesis of the α-globin gene requires the 3'UTR sequence of these genes to destabilize the ζ-globin mRNA and stabilize the α-globin mRNA selectively in concert with the transcriptional silencing of the ζ-globin gene. Both the 3'UTR sequence of ζ-globin and α-globin mRNA contain a polypyrimidine-rich element (PRE), but they differ in sequence and binding affinity to different RNA-binding proteins. The disassociation constant for α-PRE and ζ-PRE is about 0.5 nM and3.0 nM respectively, a difference of six-fold (Russell et al., 1998). The reduced affinity of RNA-binding protein complex for the ζ-PRE allows rapid degradation of ζ-globin mRNA via deadenylation. It is also observed that the poly(A) tails of the ζ-globin mRNA are shorter than those of α-globin mRNA, which may also contribute to the rapid decay

of the ζ-globin mRNA (Russell et al., 1998). This is one of the elegant examples of how differences in RNA-protein binding affinity control an important physiological process during development.

1.2.6.1.2 Cytokines expression profile in newborns versus adults

GM-CSF plays a very important role in generation of immune responses against infection by modulating myeloid activation, proliferation and differentiation of neutrophils, monocytes and platelets. The expression of GM-CSF mRNA and protein in activated neonatal mononuclear cells (MNCs) from umbilical cord blood are about seven and four fold less compared to adult peripheral blood MNCs (Buzby et al., 1999). The expression of GM-CSF is partly controlled by the rate of the mRNA decay, which is regulated by the ARE sequence within the 3'UTR region of the GM-CSF mRNA (Shaw et al., 1986). The transcription rate of GM-CSF is very similar in the MNC to the two groups, but the half-life of the GM-CSF mRNA is three-time shorter in the neonatal MNC (19 min as oppose to 79 min in the adults MNC) (Buzby et al., 1999). The rapid decay in vitro is both ARE and RNA-binding protein AUF1 dependent. Wagner et al were able to show that the p37 isoform of AUF1 is predominately expressed in neonatal MNC, whereas the p45 AUF1 is exclusive to adults MNC (Wagner et al., 1998). They were also able to show that p37 possesses a higher ARE-binding affinity than p45 isoform, which promote rapid ARE-bearing mRNA degradation in the neonatal MNC (Wagner et al., 1998). The half-life of other cytokines such as MIP1α, IL-3 IL-8 and IL-12 are shorter in neonatal MNC as well. Thus, mRNA stability can play a major role in age dependent immune functions.

1.2.6.1.3 Control of c-myc proto-oncogene during differentiation

As mentioned before, the embryonic development and cellular differentiation are totally regulated by the coordinated control of gene expression and cellular proliferation. One of the

most intensely studied master regulators of gene that has a significant influence on cellular proliferation and differentiation is the *c-myc* transcription factor. Experiments in the human K562 cell line are able to show elegantly how the *c-myc* silencing controls the fate of differentiating into the erythroid or the platelet precursors during development. The activation of protein kinase C (PKC) signaling pathway in the K562 cells reduces the half-life of the *c-myc* mRNA by four fold, resulting in 90% decline of *c-myc* mRNA, which leads to loss of erythroid properties and differentiation into megakaryoblast (Brewer, 1998). The PKC induced destabilization of *c-myc* mRNA is mediated by a deadenylation-independent pathway.

1.2.6.1.4 Iron-responsive element (IRE) and iron-regulatory protein (IRP)

The 3'UTR of the transferring receptor (TfR) contains five iron response elements (IRE), three of which are reported to regulate the mRNA half-life, another one is located in the 5'UTR and is known to affect translation. These IREs interact with two specific iron-regulatory proteins known as IRE-binding proteins (IRP1 and IRP-2) and modulate the intercellular iron concentration by assembling an iron-sulfur cluster in IRP1 and stabilizing the IRP2 protein. The IRP responds to low intercellular iron concentration in two different ways, (i) it interacts with the three IREs located at the 3'UTR of the TfR mRNA, thereby stabilizing it and allow the iron uptake, (ii) it also can bind to the IRE in the 5'-UTR of the ferritin mRNA, an iron sequestration protein, thereby blocking the translation initiation and preventing iron sequestration. On the other hand, when the iron concentration is very high, the iron-sulfur cluster in the IRP1 is disassembled and the IRP2 is a target for ubiquitin-directed proteolysis. This inhibits the interaction between IRPs and TfR mRNA, which then becomes more accessible for endonucleolytic cleavage, thus limiting iron uptake (reviewed in Hardie et al., 1998).

1.2.6.1.5 mRNA stability during replicative-senescence

The AMP-activated protein kinase (AMPK), is an enzyme that works as a metabolic sensor for low fuel status in the cell (Hardie et al., 1998). The AMPK is activated by the high concentration of AMP and is inhibited by the elevated levels of ATP. The AMP:ATP ratios are 2-3 fold higher in the high-passage senescent old fibroblasts compared to young fibroblast, which leads to the activation of the AMPK pathway. By using the *in vitro* human IDH4 fibroblast model system, Wang and his colleagues were able to show that AMPK influences the ARE-containing mRNA turnover by inhibiting the cytoplasmic export of the RNA-binding protein HuR (Wang et al., 2002). As mentioned previously, the HuR is predominantly a nuclear protein (>90%) in the unstimulated cells and cytoplasomic translocation is necessary for the HuR to stabilize ARE-mRNAs. The AMPK-induced inhibition of the HuR migration and decreased cytoplasomic mRNA turnover of many different prolifirative genes including cyclins A, and B1, c-Fos and DP-1 have been observed (Jain et al., 1997). They were also able to show that aged or high passage cells express less total HuR (both cytoplasmic and nuclear). The combined effects of both decreased expression and cytoplasmic localization of HuR allow faster decay of these mRNAs (Wang et al., 2003a). The aged cells contain decreased levels of HuR dependent ARE-RNA-binding and stabilizing activity compared to young cells. Interestingly, the level of another AUBP AUF1 also decreases with age, which is shown to increase the stability of cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p16^{INK4a}$, which may contribute to a cell-cycle arrest and induce replicative senescence (Wang et al., 2005).

1.2.6.2 Deregulated mRNA stability: A diseases mechanism?

As repeatedly mentioned throughout this review, many of the early and transient response genes are regulated at the level of mRNA stability by the ARE located in the 3'UTR of their

mRNA, which is critical for controlling important biological responses such as cell growth, cell cycle regulation and apoptosis, host defense, angiogenesis, ionic homeostasis and responses to exogenous agents like radiation, virus and inflammatory stimuli. A recent report showed that ARE-mRNA represents 8% of all the mRNAs transcribed from functional human genes (Khabar, 2005). So the deregulation in the ARE-mediated control of gene expression could have a dire consequence in maintaining a normal physiology that lead to undesirable pathologic states. Following are few examples of human disease conditions resulting from unwanted changes in ARE-mediated events.

1.2.6.2.1 α-Thalassemia

α-Thalassemia is a disease that arises from decreased stability of the normally very stable α-globin mRNA by the presence of an anti-termination sequence. The expression of goblin genes is regulated both transcriptionally and post-transcriptionally. The diseased alleles of the α-globin gene contain termination mutation of UAA to CAA that allows translating ribosomes to proceed into the 3'UTR, which mask the binding sites for stabilizing protein at the C-rich regions of the 3'UTR. By preventing the interaction between the RNA-binding proteins and the α-globin mRNA, the poly(A) undergoes rapid shortening and premature degradation of mRNA, which leads to α-thalassemia (Forget, 1979).

1.2.6.2.2 Myotonic Dystrophy

Myotonic dystrophy (DM) is a dominant inherited multi-system disorder, which is caused by an expanded number of trinucleotide (CTG) repeats in the 3'UTR of a cAMP-dependent protein kinase gene (DMPK). Most of the normal alleles of the DMPK gene contain 5-30 repeats, but with the most severe forms of disease, characterized by respiratory distress, endocrine dysfunction and mental retardation, the number of repeats reaches 1000 or more (Lu

et al., 1999). The mutated mRNA is thought to bind to an ELAV-like ribonucleoprotein and is retained within the DM myoblast nuclei, which leads to impaired kinase synthesis and reduces its function in ion channel phosphorylation (Davis et al., 1997) that is thought to be cause of change in the muscle excitability.

1.2.6.2.3 Alzheimer's Diseases (AD)

In the brain of Alzheimer's patients, iron cannot be properly sequestered by ferritin, which leads to an excessive iron accumulation, cells therefore become vulnerable to oxidative stress (Percy et al., 1998). The iron homeostasis in normal physiological conditions is achieved by the simultaneous regulation of ferritin, an iron sequestration protein, and the transferrin receptor (TfR), which is the iron uptake protein. These two proteins allow the cell to obtain iron when needed and sequester it when in excess. It has been hypothesized that a more stable form of iron-responsive element/ iron-regulatory protein complex in the Alzheimer's patient brains could stabilize the TfR mRNA while inhibiting ferritin synthesis (Guhaniyogi & Brewer, 2001). Such alteration would result in an increased iron uptake without appropriate sequestration of iron by ferritin.

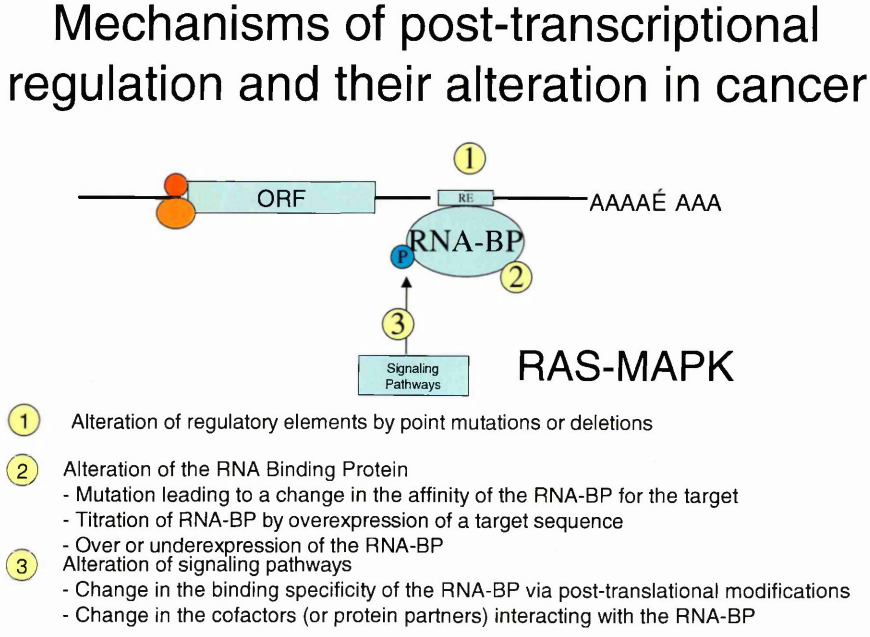
Another AD related gene that has received much attention is the amyloid precursor protein (APP) (Rajagopalan et al., 1998). APP synthesis is deregulated in the AD patient brains and APP deposits of extracellular α-amyloid within the central nervous system in the AD patients were observed. The transgenic mice that overexpress APP has accelerated deposition of α-amyloid. Elevated levels of APP mRNA was detected in the brain tissue of AD patients. A 29-nt, C+U-rich sequence located approximately 200-nt downstream of the stop codon in the 3'UTR of the APP mRNA is required and sufficient to control the stability of the APP mRNA (Zaidi & Malter, 1994). It has been reported that the stabilization of APP mRNA in

the AD patients is regulated by heterogeneous ribonucleoprotein C (hnRNP C) and its interaction with the U-rich sequences in the 3'UTR of the APP mRNA may contribute to elevated APP levels in the AD (Rajagopalan et al., 1998).

1.2.7 mRNA stability in cancer

The fundamental abnormality resulting in the development of cancer is the continual unregulated gene expression that leads to an uncontrolled proliferation of cancer cells. The genes required for the maintenance of the abnormal cell growth are known as oncogenes. Oncogene overexpression is one of the hallmarks of cancer; cancer cells utilize variety of different molecular pathways to achieve that. A most efficient and economic way of regulating gene expression is controlling the stability of the transcribed mRNAs, which has been exploited by the cancer cell very efficiently. The stability of the many different ARE bearing mRNAs including poto-oncogenes and growth factors are significantly altered by the presence of the unregulated *trans*-acting factors in the cancer cell, leading to the cancer causing protein overexpression and cellular transformation.

Figure 1.8



The regulation of mRNA stability is controlled by a complex network of RNA/RNA-binding protein interactions, which are influenced by the signal transduction pathway that can post-translationally modify RNA-BPs and the interaction with the target mRNA. The mechanisms of altering the mRNA stability and gene regulation in cancer cells can be divided into three major groups: (i) altering mRNA stability by point mutation or deletions in the regulatory element of the mRNA, (ii) modification of the RNA/RNA-BPs interaction by mutations in the RNA-BPs which change the affinity of the RNA-BP for its target mRNA or by over/underexpressing the RNA-BPs, and (iii) alteration of signaling pathways that affect the RNA/RNA-BPs interaction (Audic & Hartley, 2004). In this part of the chapter I will briefly review the mechanisms of altering the mRNA stability as a means to up or downregulate gene expression in cancer.

1.2.7.1 Alteration of regulatory elements in the 3'UTRs:

Although the regulatory sequence that influences the mRNA stability can be found in the all-different parts of the mRNA, the elements located in the 3' untranslated region are considered to be the most important mRNA stability determinants. From a mechanistic point of view, any RNA/protein interaction that takes place at the 3'UTR, which determines the stability of the mRNA, can persist throughout the translation, because it is very unlikely that ribosomes scan the 3'UTR region (Poyry et al., 2004). So the regulatory elements in the 3'UTR can influence the mRNA stability at any time. We will discuss few examples of how alterations in regulatory sequence can promote tumorigenesis.

1.2.7.1.1 Cyclins D

A fundamental aspect of cancer is the impaired cell cycle checkpoint. Unlike normal cells that only proliferate in the presence of mitogenic signals in response to the

extracellular stimuli or during development. The proliferation of cancer cell is uncontrolled due to the alteration in the many aspects of the cell cycle. The endpoint of these alterations is the inappropriate proliferation commonly associated with carcinogenesis. Cyclin and cyclin-dependant kinases (cdks) complexes are central for the progression and control of the mammalian cell cycle (Malumbres & Barbacid, 2001). The activation of each cdk is controlled by the availability of a cyclin partner and a complex network of post-translational modification of these proteins, which then control the progress from one cell cycle phase to other. The expression of different cyclins is regulated by the phase of the cell cycle; cell cycle dependent mRNA stability plays an important role in controlling the cyclin expression. D-type cyclin (D1, D2 and D3) expression is highest in the early G1 phase where they interact and activate cdk4 and cdk6. The complexes then phosphorylate the Rb tumor suppressor and release E2F transcription factors to initiate the S-phase of cell cycle.

The 3'UTR elements of the cyclin D1 and D3 mRNA can regulate the mRNA turnover and gene expression in response to different extracellular stimuli (Langenfeld et al., 1997). Several human cancers are known to overexpress the cyclin D1 gene (Donnellan & Chetty, 1998). Rearrangement in the 3'UTR of the cyclin D1 gene have been reported in patients with mantle cell lymphomas and t(11q13)-associated leukemia, ((Tsujimoto et al., 1985) (Nakagawa et al., 2006). In both cases, deletion of the AU-rich region of the 3'UTR is responsible for stabilizing the cyclin D1 mRNA, which leads to an overexpression of cyclin D1protein. The half-life of the rearranged cyclin D1 mRNA is greater than 3 hours compared to 0.5 hour for cyclin D1 mRNA from normal tissue (Rimokh et al., 1994). A similar rearrangement is also reported in the 3'UTR region of the cyclin D1 in

neuroblastoma (Molenaar et al., 2003). MDA MB-453 a human breast cancer cell line, contained cyclin D1 mRNA where the AU-rich region is deleted. This truncated cyclin D1 mRNA is more stable than the full length mRNA and is considered to be the cause of the cyclin D1 overexpression in the MDA MB-453 cell line (Lebwohl et al., 1994).

Prostaglandin A_2 (PGA_2) is an experimental chemotherapy that causes cell cycle arrest in several human tumor cell line by decreasing the cyclin D1 expression (Gorospe et al., 1996). Cells treated with PGA_2 are shown to upregulate ARE-binding protein AUF1, which leads to a decrease in stability of the cyclin D1 mRNA through a specific 390 bp region in the 3' UTR (Lin et al., 2000). Glucocorticoids is another chemotherapeutic agent that is know to inhibit cyclin D3 expression and induces a G0/G1 cell cycle arrest in the T lymphoma cells by destabilizing its mRNA (Reisman & Thompson, 1995). These examples highlight the potential importance of mRNA stability in cancer development and also in cancer therapy.

1.2.7.1.2 C-myc

C-myc is a master transcription factor known to dimerize with its cellular partner, binds to E-box DNA and transactivate many different genes in different stages of the development (Levens, 2002). In normal cells c-myc mRNA is extremely labile. The stability of c-myc mRNA is controlled by three different elements, which include AREs in the 3'UTR that control instability (Herrick & Ross, 1994) a coding region elements (Wisdom & Lee, 1991) and a 5'UTR internal ribosomal entry site (Nanbru et al., 1997), which controls translation. Deregulated c-myc expression is associated with cancer, which supports many aspects of tumorogenesis including proliferation, growth, metabolisms and differentiation. Both deletion (Aghib et al., 1990) and translocation in the 3'UTR region of the c-myc gene

(Hollis et al., 1988) have been reported in human T-cell leukemia and human myeloma cell line respectively. These observations suggest that the deletion in the 3'UTR region may be playing a role in the cancer cell selective c-myc mRNA stabilization and overexpression of c-myc proteins. But, transgenic mice carrying a similar deletion in the c-myc gene show no phenotype and the half-life of the c-myc mRNA was normal (Langa et al., 2001). It has been speculated that beside mRNA stability, another mechanisms may be involved in deregulating the c-myc expression in cancer cells.

1.2.7.2 Alteration in the RNA binding factors

AREs facilitate the binding of cellular RNA-binding proteins that recruit other proteins at the site and guide degradation or stabilization of the target mRNA. The overall protein production from a specific mRNA is ultimately determined by the competition between the stabilization and degradation factors binding at the site of the ARE of this mRNA. In the human genomes about 500 proteins have been identified to contain RNA-binding domain (Anantharaman et al., 2002). At least 14 of these proteins known to alter their activity in cancer in various ways, which than affect the stability of different ARE-containing mRNAs. We will discuss a few well characterize RNA-binding proteins that known to play a significant role in cancer development.

1.2.7.2.1 HuR

Human-antigen R (HuR), is one of the best characterized RNA-binding proteins. It was first isolated as a tumor antigen in lung carcinoma of individuals with paraneoplastic neurological disorder (Szabo et al., 1991). HuR is predominately a nuclear protein and

thought to bind to ARE containing mRNAs in the nucleus and export through the nuclear pores to the cytoplasm and induced mRNA stabilization (Fan & Steitz, 1998).

Increased expression of cyclooxygenase-2 (COX-2) has been observed in several human tumors (Thun et al., 2002). COX-2 affects many aspects of carcinogenesis including angiogenesis, apoptosis, immune escape and tumor invasiveness (Gupta & Dubois, 2001). Different oncogenes, cytokines, growth factors and tumor-associated genes known to induce COX-2 expression by activating transcription and also by enhancing mRNA stability transiently (Ristimaki et al., 2002). In human colon cancer, Increased HuR expression is directly correlated with the COX-2 expression (Dixon et al., 2000). Increased binding of HuR to a conserved AU-rich element in the 3'UTR region within the COX-2 mRNA decreased COX-2 mRNA decay and increased COX-2 protein levels. Other ARE-containing, tumor promoting factors such as angiogenic factor VEGF and proliferative factors IL-8 also upregulated in colon and brain tumor cells presumably due to mRNA stabilization by the overexpressed HuR protein (Dixon et al., 2000). Cyclin A and B1 play a central role in controlling the cell cycle progression from the S to the G2 phase and their expression are tightly regulated during the cell division. The post-transcriptional regulation by mRNA stability is very important for the regulated expression of these proteins (Maity et al., 1995). During the S phase, the HuR protein localizes from nuclear to cytoplasm and selectively stabilizes the cyclin A and B1 mRNA (Maity et al., 1997). In the RKO colorectal cancer cell, the antisense mRNA mediated inhibition of the HuR expression leads to a reduced half-life of the cyclin A and B1 mRNA and an inhibition of cell growth and proliferation (Wang et al., 2000a). These results illustrate the importance of HuR in the cell cycle regulation by post-transcriptional mechanisms.

1.2.7.2.2 Tristetraprolin (TTP)

It has been reported that the defects in the ARE-mediated mRNA stabilization result in an increased expression of the autocrine hematopoietic growth factors GM-CSF, which is known to contribute to the pathogenesis of leukemia (Hoyle et al., 1997). The ARE-binding protein tristetraprolin has been shown to regulate the GM-CSF mRNA stability by interacting with the AU-rich region of the ARE and induce deadenylation mediated mRNA decay (Schuler & Cole, 1988). The GM-CSF mRNA is markedly stabilized in the TTP knockout transgenic animals (Carballo et al., 2000), which establishes a clear role of TTP in regulating GM-CSF expression via mRNA stabilization.

In a human colorectal cell line HCA-7, two different COX-2 mRNA with different lengths of 3'UTR have been identified. One of the mRNA with a longer 3'UTR contains the binding site for the TTP and is subject to TTP mediated mRNA degradation. The COX-2 mRNA with the shorter version of 3'UTR without the TTP binding site cannot be targeted by the TTP for degradation and is responsible for a sustaining elevated level of COX-2 expression in the cancer cells (Boutaud et al., 2003).

1.2.7.2.3 AUF1

AUF1 is an ARE-binding protein that is shown to cause mRNA destabilization. The target mRNAs for AUF1 encode many important proteins that are involved in mitogenic signaling, immune response, cancer-associated and cell-cycle regulation. As mentioned before, this protein is expressed in four isoforms (p37, p40, p42 and p45) arising from the same mRNA by alternative splicing (Wilson et al., 1999). In vitro, the p37 isoform has the highest affinity for the AREs and is most efficient in destabilizing the ARE-mRNA (Loflin et al., 1999). The p37 AUF1 overexpressing transgenic mice develop sarcomas by

upregulating the cyclin D1 expression (Gouble et al., 2002). Elevated levels of interleukin-10 (IL-10) production were observed in the malignant melanoma cells, which is responsible for inhibiting immune surveillance and tumor rejection (Steinbrink et al., 2002). The IL-10 mRNA half-life of the melanoma cells increases dramatically compared to normal melanocytes (75 min. vs 7 min.). This abnormal stabilitization of IL-10 mRNA in the melanoma cell appears to be due to the very low cytoplasmic levels of AUF1 (Brewer et al., 2003).

1.2.7.3 Alteration in the signaling pathways and mRNA stability

The third most important component of the ARE-mediated regulation of mRNA turnover is comprised of the intracellular cell signaling pathways. Compared to our current understanding of how intracellular signaling regulates cellular processes such as transcription, very little is known about the signaling events regulating mRNA stability. However, several reports have provided increasing support for signal transduction pathways as being possibly involved in regulating the mRNA stability. The following are the few examples of the best characterize signaling pathways that are involved in regulating stability of the different ARE-mRNAs.

1.2.7.3.1 p38 MAPK signaling

The p38 MAPK pathway has been shown to regulate the half-life of a number of ARE-bearing mRNAs including COX-2, IL-3, IL-6, IL8, TNF-α, VEGF, GM-CSF, c-fos and uPA (Dean et al., 2004). Extracellular ligands such as different pro-inflammatory stimuli can bind to the target receptor and activate upstream MAP kinases such as MKK6 and MKK3, which then activate the p38 MAPK and regulate the stability of the p38 responsive

mRNAs. More than 40 ARE-mRNAs have been recently reported as potential targets for the p38 MAPK pathway (Frevel et al., 2003). The ARE-mediated deadenylation directly correlates with the p38 MAPK activity. The inhibition of p38 MAPK pathways leads to a reduction of mRNA decay and an increase in translation efficiency (Dean et al., 2003). Several studies identify the MK2 as the key downstream effectors molecule in the p38 mediated mRNA stabilization. Both the endotoxin-induced COX-2 mRNA stabilization and the COX-2 expression can be abolished by expressing dominant-negative MK2 or inhibition of the p38 pathway using pharmacological inhibitor SB203580 (Dean et al., 1999). The COX-2 expression is linked to poor prognosis of many human cancers. Also treatment with the selective COX-2 inhibitor celecoxib reduces the number of colorectal polyps in patients with familial adenomatous polyosis (Phillips et al., 2002). The p38 MAPK pathway has been reported to play an important role in the cancer cell selective COX-2 mRNA stabilization and COX-2 induction. In HeLa cell, IL-1 induced COX-2 expression can be reduced by an inhibition of p38 signaling by destabilizing the COX-2 mRNA (Ridley et al., 1998). In Barrett's esophagus, acid-activated p38 MAPK stabilized COX-2 mRNA and increases the COX-2 expression, suggesting potential mechanisms whereby acid reflux might promote carcinogenesis (Souza et al., 2004).

The transforming growth factor-β1 (TGF-β1) is a member of a large family of multifunctional polypeptides that promotes tumor growth, immune suppression, angiogenesis and metastasis (Stearns et al., 1999). Both the intercellular and serum levels of TGF-β1 are elevated in prostate cancer patients and even more so in patients with metastasis (Eastham et al., 1995). In the prostate cancer, TGF-β1 can elevate the expression of ARE-binding protein HuR (Park et al., 2003), which binds to ARE elements within the

3' untranslated region of the tumor-promoting cytokine IL-6 mRNA and stabilize the mRNA. The inhibition of the p38 signaling pathway abrogate TGF-β1–induced IL-6 mRNA expression (Park et al., 2003). Matrix metalloproteinase protein-9 is involved in disrupting the basement membrane during angiogenesis and tumor invasion. The TGF-β1 induces MMP-9 mRNA stabilization and elevated MMP-9 expression is reported in different malignancies (Sehgal & Thompson, 1999).

The urokinase-type plasminogen activator (uPA) interacts with its receptor (uPAR) to promote cell migration as well as proliferation and contributes to the pathogenesis of neoplastic growth and invasiveness. The expression of both uPA and its receptor at the tumor sites correlates with poor prognosis of cancer. In the invasive breast cancer cells, constitutive p38 MAPK activity is essential for uPA expression by promoting stability of the ARE-containing uPA mRNA (Han et al., 2002). The expression of the dominant negative MK2 inhibits uPA expression as it destabilizes the uPA mRNA.

The precise mechanism of how the p38 pathway regulates the ARE-containing mRNA turnover remains unknown. Hypothetically, RNA-binding proteins could be an ideal target for the post-translational modification by the p38 pathway, which eventually leads to alteration in their ability to stabilize or destabilize ARE-mRNAs. In recent years, there has been much progress in identifying RNA-binding proteins that are affected by the intercellular signaling. One such example is the p38-mediated modification of the mRNA binding protein tristetraprolin (TTP). TTP exists in numerous phosphorylated forms and known to be targeted by several signaling pathways including p38. The hypophosphorylated form of TTP binds to ARE sequences more efficiently than the

hyperphosphorylated form (Carballo et al., 2000), which alters its ability to destabilize ARE-mRNAs.

1.2.7.3.2 Wnt/β-catenin pathway

The wnt/β-catenin pathway has been extensively linked to cancer (Kikuchi et al., 2006). This pathway rapidly induces expression of cell type specific transcription factor Pitx2, cyclins D1 and D2 by stabilizing the mRNA of these genes. Wnt activation induces the cytoplasmic localization of the ARE-binding protein. Wnt-induced Pitx2 mRNA stabilization is due to a decreased interaction of its ARE with the destabilizing ARE-BPs like TTP and an increased interaction with the stabilizing ARE-BPs HuR (Briata et al., 2003).

1.2.8 MicroRNA and the 3'UTR mediated mRNA stability:

MicroRNAs (miRNAs) represent a new class of highly conserved, about 22-nucleotide non-coding RNAs that are thought to negatively regulate the expression of protein-coding genes by targeting mRNAs for cleavage or translation repression (Bartel, 2004). By using bioinformatics prediction and molecular cloning strategies, hundreds of miRNAs have been identified in worms, flies, fish, plants and mammals (Ambros et al., 2003). The primary miRNAs (pre-miRNAs) are first transcribed in the nucleus to produce stem-loop structure of about 80nt (Bartel, 2004) and immediately processed by the RNase III enzyme Drosha, which excises the stem-loop from pre-miRNA (Bartel, 2004). This pre-miRNA is then exported from the nucleus to cytoplasm by export receptor Exportin-5 (Bartel, 2004) and are further processed by another RNase III endonuclease, Dicer, to generate the mature miRNAs as part of a short RNA duplex. The mature miRNA is then subsequently unwound by a helicase-like enzyme and is incorporated into a RNA-induced silencing complex

(RISC), where they can direct RISC to downregulate target gene expression by either mRNA cleavage or inhibition of effective mRNA translation (Bartel, 2004). The mammalian miRNAs are thought to inhibit gene expression by repressing productive translation through imperfect complementary sequences with the 3'-UTR of the target mRNAs (Doench et al., 2003). Hundreds of miRNAs have been identified, but their targets are yet to be determined. But theoretically based on bioinformatics approach to predict miRNA targets, a given miRNA can target one to hundreds of different genes. About 1-5% of all animal genes are comprised of miRNA, making it one of the most abundant classes of regulators (Bartel, 2004).

In a recent study, Jiahuai Han and colleagues observed that miR16, a human miRNA containing an UAAAUAUU complementary sequence to the ARE sequence of the TNF-α mRNA, is required for ARE-mediated mRNA turnover (Jing et al., 2005). An overexpression or inhibition of miR16 expression decreases or increases, respectively, the stability of a reporter RNA containing AREs (class II ARE containing multiple AUUUA repeats) of both TNF and COX-2, but has no effect on the reporter RNA containing uPA ARE (class III ARE with no AUUUA repeats). The miR16-mediated ARE-mRNA decay is sequence-specific and the ARE-binding protein tristetraprolin (TTP) plays an essential role in the process ARE-mRNA degradation. By using very elegant knockdown experiments in both Drosophila and mammalian systems with the siRNA, they were also able to show that many cellular components involved in the miRNA-mediated regulation gene expression are required or even essential for ARE-mediated RNA degradation, suggesting these two mechanisms of gene regulation are somehow interconnected. Comprehending the relationship between these two pathways of gene regulation will not only be important for

improving our knowledge of regulation of gene expression globally, but also it will give us new insights about their involvement in human diseases. For example, it has been reported that a cluster of two miRNAs, miR-15 and miR16-1 located on chromosome 13q14.3, is most frequently deleted genomic region in human chronic lymphocytic leukemia (CLL), which leads to a downregulation of both genes in the majority of CLL cases (approximately 68%) (Calin et al., 2005). The antiapoptotic protein bcl-2 expression in CLL is inversely correlated with the miR15-a and miR16-1 expression and both of the miRNAs negatively regulate bcl-2 at a posttranscriptional level. Many other genes (e.g. MRP, COX-2) have been reported to overexpress in CLL by increasing the stability of their mRNA and are important for the tumorogenesis (Ryan et al., 2006). Based on these reports, it might not be premature to speculate that miRNAs like miR16, may be an important component of the ARE-mediated mRNA decay.

1.2.9 Conclusion and future direction

The mechanism of mRNA stability as a means to regulate gene expression is found in all living organisms. The kinetics of mRNA accumulation following transcription is determined by the stability of the mRNA, which is one of the key factors that control the quantity of protein produced from that mRNA. A given mRNA may also be controlled by several post-transcriptional mechanisms, such as microRNA or mRNA stability, in order to achieve the appropriate expression in both time and space. The ARE-mediated mRNA turnover is an important component of the post-transcriptional process of gene regulation, which is control by a dynamic equilibrium among *cis*-acting elements in the mRNA, RNA-binding proteins and the signaling pathways that are capable of modifying the interaction between mRNA and RNA-BPs in a particular cellular environment.

Compared with the amount of knowledge we have in regards to the regulation of gene expression by transcription, our understanding on the post-transcriptional regulation of gene expression is still in its infancy. Many questions remain to be answered. There is very little information about the secondary structure of the ARE and how that may play a role in the mRNA turnover. Increasing knowledge on the structure of ARE will be very important for better understanding of the interaction with the RNA-binding proteins and the ARE within the 3'UTR of the target mRNA. Many RNA-BPs seem to shuttle between the nucleus and cytoplasm; different cellular signaling pathways have been shown to modulate this process. It has become clear that some signaling events modify specific RNA-BPs by phosphorylation, which controls their interaction with the AREs. But the relationship between intracellular signaling and the compartmentalization of the RNA-BPs is not well understood. We also need to address the role of microRNAs in the process of ARE-mediated mRNA decay. Recent reports have identified cellular factors that are involved in both of these regulatory processes. It is yet to be determined how these two post-transcriptional pathways of gene regulation are interconnected. And finally, as pointed out in this review, several human diseases are caused by the defects in the process of mRNA turnover. In-depth knowledge about the post-transcriptional regulation of gene expression will provide a solid ground for designing effective therapy for these diseases.

1.3 Cyclooxygenase-2

1.3.1 Introduction:

Aspirin has been used as an analgesic and anti-pyretic medicine for over a century with very little knowledge about its mechanisms of action. In the late sixty's, Samuelsson and

Bergstrom discovered the prostaglandin (PG) synthesis pathways (Nugteren et al., 1966) and few years later JR Vane and his colleagues identified cyclooxygenase (COX) as the molecular target for Aspirin, the rate-limiting enzyme for prostaglandin biosynthesis (Ferreira et al., 1971). In 1982 the Noble committee acknowledged the importance of this discovery by awarding Drs. Vane, Samuelsson and Bergstrom the Noble Prize for Physiology and Medicine. Now a family of drug exists with the similar properties, collectively known as non-steroidal anti-inflammatory drugs (NSAIDs).

The enzyme COX exists as two distinct isoforms, COX-1 and COX-2. COX-1 is constitutively expressed as a housekeeping enzyme in most of the tissues and controls normal physiological functions such as platelet aggregation, and regulation of renal blood flow (Smith et al., 2000). By contrast, COX-2 is expressed by cells that are involved in inflammation and responsible for the synthesis of the PGs associated with pain and fever. The suppression of COX-2 activity is thought to be the main therapeutic action of NSAIDs, on the other hand the inhibition COX-1 results in unwanted side effects such as ulceration, bleeding, and obstruction at the gastrointestinal tract (Smith et al., 2000). The expression of COX-2 has been reported to be elevated in many different human cancers including colorectal, breast, cervical, prostate and lung (Zha et al., 2004). Multiple lines of epidemiological evidence indicated that the use of COX-2 inhibitor NSAIDs is associated with a reduced risk of several malignancies (Zha et al., 2004). Also, transgenic animals with knockout COX-2 or animals treated with selective COX-2 inhibitor, tumor formation and growth are significantly reduced, indicating a clear association between COX-2 expression and carcinogenesis (Oshima et al., 1996). Here we review the fundamental

properties of COX enzyme, especially COX-2 as related to tumorogenesis and discuss the proposed mechanisms behind their roles in cancer.

1.3.2 Gene, enzyme and structure:

The human gene encoding COX-1 is located on chromosome 9 (9q32-9q33.3), which contains 11 exones spreads across 40 kb and its mRNA is approximately 2.8 kb (Smith et al., 2000). The gene encoding COX-2 is located on chromosome 1 (1q25.2-25.3) and contains 10 exones with a 4.5 kb transcript (Smith et al., 2000). Although the two members of COX family enzymes are very different in their genomics structure and transcript size, the proteins for both enzymes are about 600 amino acids with the calculated molecular weight of about 68 kDa. But after the post-translational modifications by glycosylation, the molecular weight becomes 75-80 kDa (Smith et al., 2000).

Both of the COX isoforms catalyze the same reaction by using the same substrate and generate the same products. The X-ray crystal structures of both of the isoforms are near superimposible with few profound differences. First of all, the isoleucine 590 in the substrate channel of COX-1 is replaced by a smaller amino acid valine in COX-2 (Kurumbail et al., 1996), which gives COX-2 larger substrate binding pocket and ability to use a broader spectrum of substrate. The isolucine/valine substitution is also thought to be the structural basis for the COX-2 selective inhibitors. Another important structural difference between these two enzymes is that COX-2 contains an additional 18 amino acids towards its C-terminus end, while COX-1 enzyme contains an insertion of extra 17 amino acids towards its N-terminus end (Smith et al., 2000). COX-2 can be found in both the endoplasmic reticulum (ER) and the nuclear envelope, but COX-1 is only localized to the

ER. It has been hypothized that the C-terminal insertion might carry a nuclear localization signal that initiate the COX-2 nuclear membrane localization (Smith et al., 2000).

As mentioned above, COX-1 is constitutively expressed with constant levels in many tissues, whereas COX-2 is an inducible enzyme, which is only expressed in response to different cellular stimuli. But the high amount of constitutive expression of COX-2 is also documented in the central nervous system, the kidney and in the seminal vesicles. The substrate for COX-2 enzyme is present in the ER and the nuclear membrane where COX-2 catalyzes the rate-limiting step for prostaglandin synthesis.

1.3.3 Prostaglandin biosynthesis:

Both COX isoforms catalyze the synthesis of prostaglandins (PGs) from arachidonic acid, which is a 20-carbon polyunsaturated fatty acid released by phospholipase A_2 from the cell membrane (**Figure 1.9**). COX catalyzes the oxidative cyclization of arachidonic acid to form the unstable intermediate PGG_2, which is then rapidly reduced to a more stable PGH_2 by the peroxidase activity of COX. Under different physiological conditions, different cell types metabolize PGH_2 differently to produce dramatically different products. For examples, PGD is usually found in the mast cells and in the brain; PGF is produced in the uterus; PGI is found in the endothelial cells; thromboxane is commonly produced by platelets and macrophages (Smith et al., 2000).

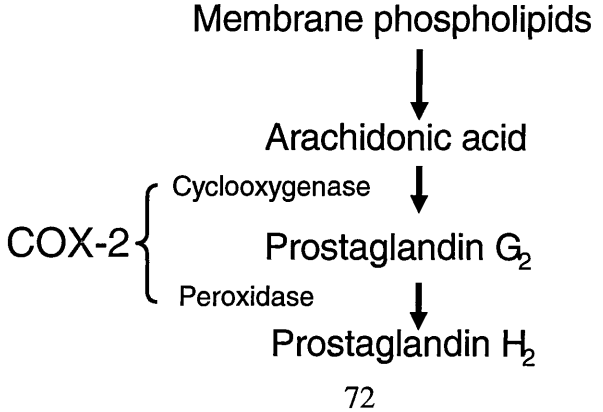


Figure 1.9 Prostaglandin biosynthesis pathway.

1.3.4 Regulation of COX-2 expression:

1.3.4.1 Transcriptional regulation

The expression of COX-2 is regulated by a broad-spectrum stimulus associated with the inflammation. The most well studied COX-2 inducers are bacterial lipopolysaccharide (LPS), pro-inflammatory cytokines such as IL-1β, TNF-α, IL-2, growth factors (e.g. epidermal growth factor, platelet derived growth factor), and some tumor promoting agents such as PMA. On the other hand, anti-inflammatory cytokines like IL-10 have been reported to inhibit COX-2 expression (Zha et al., 2004). The promoter of the COX-2 gene contains a TATA box and binding sites for several transcription factors including activator protein 1(AP-1), nuclear factor κB, nuclear factor for activated T cells (NFAT), which can modulate the transcription of COX-2 (Smith et al., 2000). As a negative regulator wild type, but not mutant, p53 markedly suppresses COX-2 transcription by competing with the TATA-binding protein for binding to the TATA-box (Smith et al., 2000). Several reports highlight the importance of the p53 status in human tumors as one of the determinant factor of COX-2 overexpression (Erkinheimo et al., 2004).

1.3.4.2 Post-transcriptional regulation

When Needleman and his colleagues first discovered the inducible isoform of the cyclooxygenase gene COX-2, they also recognized that the COX-2 expression was temporally regulated at both transcriptional and post-transcriptional levels (Raz et al., 1989). Then the identification of multiple regulatory elements within the 3'UTR of the COX-2 mRNA solidified the notion that COX-2 might be regulated at the post-transcriptional levels. The 3'UTR of the human COX-2 gene is located in the exon10,

which contains 22 copies of the AUUUA Shaw-Kamen sequences (Gou et al., 1998). The post-transcriptional regulation of the COX-2 mRNA is dependent on these elements since its presence induces rapid decay of a naturally stable reporter mRNA (Dixon et al., 2000). This AU-rich region (ARE) is highly conserved among human, mouse, rat chicken, pig and sheep COX-2 transcript, implying that the function of these regulatory elements has been conserved throughout the evolution. I will discuss later in this chapter how all the trans-acting regulatory elements and the signal transduction pathways interact with the 3'UTR and initiate post-transcriptional regulation of the COX-2 expression.

1.3.5 Physiological and pathophysiological functions of COX-2

1.3.5.1 COX-2 in pain management

COX-2 is constitutively expressed in the dorsal horn of the spinal cord and becomes upregulated briefly after trauma and during inflammation. This causes an increased synthesis of the COX-2-dependent PGs, which sensitize peripheral nociceptor terminals and produces localized pain. Experimental evidences suggesting that the increased COX-2 expression in the spinal cord may facilitate transmission of the nociceptive input. The specific COX-2 inhibitor celecoxib inhibits the inflammation-induced PGs synthesis in the cerebrospinal fluid and suppresses pain sensation (Smith et al., 1998).

1.3.5.2 COX-2 in kidney function

The kidney is one of the organs that have been reported to express COX-2 constitutively. In the human kidney, the COX-2 expression is observed in the renal vasculature, medullary interstitial cells and in the macular dense by immunohistochemistry. The limited evidence in humans indicated that the COX-2 is involved in the sodium regulation and the kidney perfusion under stress, but do not contribute in maintaining the

basal renal blood flow (Khan et al., 2001b). Various clinical studies clarify the involvement of COX-2 in human renal function and show that specific inhibitors cause peripheral edema, hypertension, and worsen a pre-existing hypertension by inhibition water and salt excretion by the kidney.

1.3.5.3 COX-2 and the cardiovascular system

It has been suggested that the COX-2 expressed by the endothelium to plays a vasoprotective and anti-atherogenic role by catalyzing the production of PGI_2, which is a potent inhibitor of platelet aggregation, activation and adhesion of leukocytes, and accumulation of cholesterol in vascular cells. In the clinical studies, specific COX-2 inhibitors have been shown to decrease systemic PGI_2 (McAdam et al., 1999).

1.3.5.4 COX-2 and the Alzheimer's diseases

The involvement of COX-2 with Alzheimer's diseases has been mostly based on epidemiological studies. A study conducted with 1686 participants showed that the risk of developing Alzheimer's disease was significantly reduced in the group of NSAIDs user. Also, COX-2 is upregulated in brain areas like the hippocampus and the cortex, which are related to memory. COX-2 expression is also correlated with the deposition of β-Amyloid protein in the Alzheimer's plaques (Xiang et al., 2002). However, the precise role of COX-2 in Alzheimer's disease is not yet known.

1.3.6 COX-2 and cancer

The idea that the COX-2 may play a role in carcinogenesis was first suggested by the epidemiological studies demonstrating the effectiveness of different NSAIDs in reducing the relative risk of clone cancer (Gupta & Dubois, 2001). Now, compelling evidence is

indicating that COX-2 is an important contributor to the process of carcinogenesis. In this part of the chapter, I will focus on two sources of evidence: epidemiological studies and results from transgenic animal models, which indicate that the COX-2 could be one of the rate-limiting factor and also will discuss proposed mechanisms for the role of COX-2 in cancer development.

1.3.6.1 Epidemiological evidence for an association between COX-2 and carcinogenesis

One of the earliest epidemiological study that evaluated the anti-cancer properties of a selective COX-2 inhibitor sulindac was reported by Dr. Willlliam Waddell and his colleagues, where they were able to show the regression of rectal polyps in a small number of familial adenomatous polyposis patients (FAP) in response to sulindac (Waddell & Loughry, 1983). This work initiated a number of additional epidemiological studies as well as clinical trials. The results from one of the randomized double-blind placebo controlled trials on FAP patients suggested that treatment with celecoxib 400 mg twice daily for 6 months reduced the number of colorectal polyps by 28% (Steinbach et al., 2000). Similar studies on other high-risk populations have shown some beneficial reduction of the number and the size of adenoma, but the effects were inconsistent (Baron et al., 2003). A number of epidemiological studies on colorectal cancer patients also showed about 30-50% reduction in risk of colorectal cancer or adenomatous polyps or death from colorectal cancer. Based on these results, the FDA approved celecoxib as an adjunctive therapy for FAP patients. These beneficial effects of NSAIDs as anti-cancer agents directly correlate with escalating doses and duration of the treatment (Giovannucci et al., 1995). Although the early results

look very promising, currently there is not enough evidence out there to draw any conclusion on the chemo-preventative value of NSAIDs.

The COX-2 overexpression in human colon cancer was first reported by the Eberhart's group in 1994. Among all the clinical colon cancer samples tested in different studies, the percentage of COX-2 positive cells varied from 40-100%. There was a great deal of variability among these studies, even though most cases are from the FAP patient group. In these patients, increased COX-2 expression correlated with larger polyp size and also the invasiveness of the polyp (Khan et al., 2001a). But it is still unclear which cell-type actually expresses COX-2 in the tumor microenvironment or when COX-2 expression is required during the tumor progression.

1.3.6.2 Genetic evidence for an association between COX-2 and carcinogenesis

The first genetic evidence of the involvement of COX-2 in the process of carcinogenesis came from a transgenic murine model of FAP (mice carrying inactive tumor suppressor gene $APC^{\Delta 716}$). When these $APC^{\Delta 716}$ mice were crossed with another transgenic model carrying an inactivated COX-2 gene, the number and the size of the intestinal polyps were reduced in a dose-dependent manner (Oshima et al., 1996). In the transgenic mice model, where COX-2 is placed downstream of the murine mammary tumor virus promoter and a high level of COX-2 expression is induced in the mammary gland only during pregnancy, development of mammary gland hyperplasia, dysplasia and metastatic tumors were observed. The overexpression of anti-apoptotic protein Bcl-2 is seen in only in the tumor of these mice. In addition to these genetic evidences, it was found that treatment with the selective COX-2 inhibitor suppressed the growth of the different tumors in the animal model, which supports the role of COX-2 in tumorigenesis.

1.3.6.3 Proposed mechanisms for the role of COX-2 in cancer

1.3.6.3.1 Support angiogenesis

Tumor angiogenesis not only supports tumor growth by supplying all the necessary nutrients, but also provides an important path for tumor metastasis. The first evidence that COX-2 is involved in tumor angiogenesis came from a study where the growth of the COX-2 positive tumor cells was suppressed in the nude mice by COX inhibitor diclofenac through blocking angiogenesis. Subsequently, numerous reports showed by immunohistochemistry that COX-2 is co-localized with angiogenic factor like VEGF, PDGF or bFGF in different cancer cells. The COX-2 up-regulation leads to prostaglandins synthesis, which has been shown to play an important role in $\alpha V\beta 3$ integrin-induced endothelial cell migration, a necessary step for tumor angiogenesis (Dormond et al., 2001). The overexpression of COX-2 also leads to the production of matrix metalloproteinase, which is known to initiate the ECM invasion. Moreover, tumor angiogenesis is thought to be a major target in the clinical trials for different COX-2 inhibitors in many cancers (Masferrer et al., 2000).

1.3.6.3.2 Anti-apoptotic

The COX-2 overexpression in the tumor microenvironment makes the tumor cells resistant to apoptosis via altering the membrane death receptor pathway and shifting the balance of the pro-apoptotic to the anti-apoptotic protein expression. Treatment of human colon cancer cells with the PGE_2 leads to increased expression of the anti-apoptotic protein Bcl-2 and reduction in the basal apoptotic rate (Sheng et al., 1998b). Also in the transgenic mice, where selective COX-2 overexpression in the mammary gland leads to mammary gland hyperplasia and metastatic tumors, reduced expression of pro-apoptotic genes Bcl-

x(L), Bax and increased levels of anti-apoptotic protein Bcl-2 were observed only in the COX-2-induced tumor tissue.

1.3.6.4 Conclusion:

In the last 15 years, our understanding of the biology of the COX-2 enzyme has begun to shed some light on the mechanisms of the pro-cancerous roles of this enzyme. But it is still not clear at what stage of carcinogenesis the COX-2 is required or expressed. Inhibiting COX-2 by NSAIDs blocked or slowed down the development of different stages of cancer, but more clinical trials are required to draw a conclusion. All these findings highlight the importance of the chronic inflammation in cancer development and that NSAIDs may become an important part of preventative care for cancer.

1.3.7 Deregulated mRNA stability and expression of COX-2 gene in cancer

Collective evidence from epidemiological studies, transgenic animal models and cell culture studies repeatedly indicates that the overexpression of COX-2 is an important step in tumorigenesis. The regulation of COX-2 expression is maintained both at the transcriptional and post-transcriptional levels and requires contribution from the multiple signaling pathways. Recent findings indicate that the increased expression of COX-2 in cancer is a combined effect of the loss of both the transcriptional and post-transcriptional regulation. This part of the chapter is intended to summarize the recent finding of deregulated COX-2 expression in cancer at the post-transcriptional levels.

1.3.7.1 3'UTR-mediated post-transcriptional regulation of COX-2 expression in cancer

The loss of AU-rich element (ARE)-mediated post-transcriptional regulation is one of the major causes of aberrant expression of different growth-associated genes in cancer

(Khabar, 2005). In the human T-cell leukemia, the deletion of the 3'UTR of c-myc proto-oncogene leads to an increase in mRNA stability and enhances oncogenecity (Aghib et al., 1990). With regard to aberrant expression of COX-2 in human colon carcinoma cells, similar findings have also been observed. Due to the alternative polyadenylation sites usage, the human COX-2 gene is able to produce two different primary transcripts (4.6kb and 2.6 kb). The 2.6 kb COX-2 mRNA is missing the distal part of the of the 3'UTR, which leads to the stabilization of its message and an increase in expression of COX-2 in the colon cancer (Sawaoka et al., 2003). This is one of the rare examples of naturally occurring alteration in the ARE region of the COX-2 mRNA resulting in deregulated protein expression in cancer. Most of the reported mechanisms of loss of COX-2 ARE function in tumor cells are primarily due to the alteration in cellular signaling or the trans-acting regulator factors that influence the post-transcriptional regulation.

1.3.7.2 Altered interaction between the COX-2 AREs and ARE-binding protein:

The post-transcriptional regulation of any ARE-containing mRNA is a dynamic process, which is maintained by the cellular signaling pathway and the interaction between various ARE-binding proteins with the AREs-mRNA. So far, eight cellular factors have been identified that bind to ARE of the COX-2 3'UTR and influence the function of the 3'UTR to promote rapid mRNA decay, increased mRNA stability or regulate translation efficiency. The role of these factors in deregulating COX-2 expression, and their importance in carcinogenesis are discussed below.

1.3.7.2.1 HuR

Both *in vitro* and *in vivo* study showed that HuR binds to COX-2 ARE and stabilizes the COX-2 transcript (Dixon et al., 2000). Elevated levels of HuR expression is the main cause

of enhanced stabilization of the COX-2 mRNA, which leads to the overexpression of COX-2 protein in colon cancer cells. Inhibiting CRM1-mediated nuclear export of the HuR protein with the anti-tumor agent leptomycin B significantly impaired the COX-2 expression (Sengupta et al., 2003). And recentstudies have shown that, an increase in cytoplasmic localization of HuR protein is shown to be associated with COX-2 overexpression and poor clinical outcome in different cancers (Denkert et al., 2004). Several recent reports indicated that an increased cytoplasmic expression of HuR is associated with a poor histological differentiation, large tumor size, and a decreased overall survival in ductal breast and ovarian carcinomas (Heinonen et al., 2005). In a study with the 83 primary ovarian carcinomas and the 9 ovarian carcinoma cell lines, a significant correlation between cytoplasmic HuR expression and the increased COX-2 expression (P=0.002) as well as histological grade (P=0.008) and mitotic activity (P=0.002) was observed (Denkert et al., 2004). Thus, HuR is the first mRNA stability protein, of which the expression in the different subcellular compartments is associated with a poor prognosis in cancer.

1.3.8.2.2 Tristetraprolin (TTP)

TTP is a zinc finger containing protein, which was originally identified as an immediate-early response gene. Extracellular stimuli induced activated ERK, p38 MAPK signaling pathway rapidly phosphorylated TTP and altered its function by reducing the affinity for the ARE-containing mRNA. In the human colorectal cancer cell line, HCA-7, TTP is shown to bind in a distal region of the COX-2 3'UTR and promote rapid degradation of full-length (4.6 kb) COX-2 transcript, whereas a 3'UTR-truncted (2.6 kb) mRNA escapes TTP-induced decay and maintains elevated COX-2 level in the tumor cells.

Although, TTP expression is downregulated in many colon cancer cells and tumor samples (Zhang et al., 1997), the exact role of TTP in deregulated expression of COX-2 in cancer is still unclear.

AUF1 is another ARE-binding protein that is known to bind the COX-2 ARE (Sully et al., 2004). A recent report demonstrated that the overexpression of AUF-1 in transgenic mice promotes sarcoma development (Gouble et al., 2002) by increasing the cyclin D1 mRNA and protein expression. Other noticeable RNA-binding proteins, known to interact with the ARE within the COX-2 3'UTR are CArG box-binding factor-A (CBF-A) (Sully et al., 2004), CUGBP2 (Mukhopadhyay et al., 2003) and T-cell intracellular antigen-1 (TIA-1). Most of the studies describing the interaction between these RNA-binding proteins and COX-2 ARE are under normal physiological condition and the mechanisms of deregulating COX-2 expression by these proteins in the tumor microenvironment are currently unknown.

1.3.7.3 Signaling pathways effecting the post-transcriptional regulation of COX-2

The majority of human tumors support their aberrant cell growth by strategically introducing genetic mutations that results in an altered signal transduction. The signaling pathways involved in maintaining the post-transcriptional regulation of genes is also targeted by the tumor cells to increase the expression of the growth-promoting genes such as COX-2. Here we discuss the signal transduction pathways that are involved in regulating the COX-2 expression in human cancers at the post-transcriptional levels.

1.3.7.3.1 Wnt/APC

Mutation in the tumor suppressor gene adenomatous polyposis coli (APC) is a common phenomenon in both sporadic and familial colorectal cancers (Cottrell et al.,

1992). The upstream of the APC gene is the Wnt mammary oncogene, secretes the signaling factor and promotes mammary cell carcinoma. The alteration in both molecules is reported in many different cancers, which then leads to a nuclear accumulation of the transcriptional activator β-catenin and the expression of a number of cancer-associated genes (Polakis, 2000).

COX-2 seems likely to be a common downstream target for the altered Wnt/APC pathways in cancer. An elevated COX-2 expression due to increased transcriptional activation was observed in Wnt transformed murine epithelial cells (Howe et al., 1999). The nuclear localization of β-catenin due to APC mutation is directly correlated with the increased COX-2 expression in the human colon cancer cells (Dimberg et al., 2001). Recent reports demonstrate that mutated β-catenin cooperates with K-RAS and synergistically stabilizes the COX-2 mRNA to promote COX-2 overexpression (Araki et al., 2003).

1.3.8. Ras signaling

Ras proteins are approximately 21-kDa membrane associated proteins, which act as a molecular switch that converts extracellular stimuli to intracellular signaling responses and modulate many important aspects of cell functions including cell proliferation, differentiation, survival and apoptosis. The membrane bound Ras proteins cycle between a GDP-bound inactive to a GTP-bound active state. The GDP/GTP cycling is regulated by a wide range of cell surface receptors those belong to receptor tyrosine kinases, G protein coupled receptor, cytokines receptors and integrins. Activated Ras interacts with more than 20 downstream effectors proteins including mitogen-activated protein kinase

(MAPK)/extracellular signal-regulated kinase (ERK1), PI3-kinase, Raf-1 serine/threonin kinase and Ral GDS.

Three different Ras genes encode three classical Ras proteins: Harvey (H)-Ras, Neuroblastoma (N)-Ras and Kirstan (K)-Ras. In humans, these genes are located on chromosome 11 (H-Ras), chromosome 1 (N-Ras) and chromosome 12 (K-Ras). These proteins display approximately 85% sequence homology and most of the differences are clustered in the C-terminal hypervariable regions. The sequence differences also correlate with functional differences of each Ras isoform. Inactivation of K-Ras is embryonically lethal, whereas both H-Ras and N-Ras knockout mice are viable (Koera et al., 1997) (Esteban et al., 2001). After the Ras proteins synthesize, they undergo posttranslational modification by prenylation and farnesylation. The appropriate posttranscriptional modification is essential for the intercellular transport of Ras proteins to the plasma membrane (Winter-Vann & Casey, 2005). H-Ras and N-Ras are targeted to plasma membrane via exocytotic pathway through the golgi apparatus; K-Ras reaches the plasma membrane by microtubule-dependent mechanisms. The fernesyl moieties of the proteins serve as a membrane anchor inside the plasma membrane where proteins become functionally active. Once activated, H-Ras and K-Ras need to translocate into the endosomal or other intracellular compartments to complete their signaling functions by activating the downstream effector molecules.

Mutated forms of Ras proteins are found in 30% of all cancers. About 90% of pancreatic cancers, 50% of colon cancers and 25% of adenomas carry K-Ras mutations. Most of these mutations are in codon 12, 13 and 61, which abolish GAPs mediated GTP-hydrolysis and cause the mutated Ras proteins to stay constantly in their active GTP-bound

state, independently of extracellular stimuli. This leads to an inappropriate signal transduction and a constitutive activation of multiple downstream MAPK signaling cascades, which initiate cellular transformation. It is becoming clear that oncogenic Ras requires cooperation from other poto-oncogene and signaling pathways such as c-myc, p53 or transforming growth factor-β (TGF-β) to promote all phases of the carcinogenesis, including the transformation of primary cells, migration, invasion and metastasis. For example, in an animal model the coexpression of c-myc with mutated Ras synergistically enhances the tumor formation (Sinn et al., 1987). The epithelial-to-mesenchymal transition is a necessary step for tumor invasion and metastasis requires cooperation between the active-Ras and the TGF-β-mediated signaling (Janda et al., 2002).

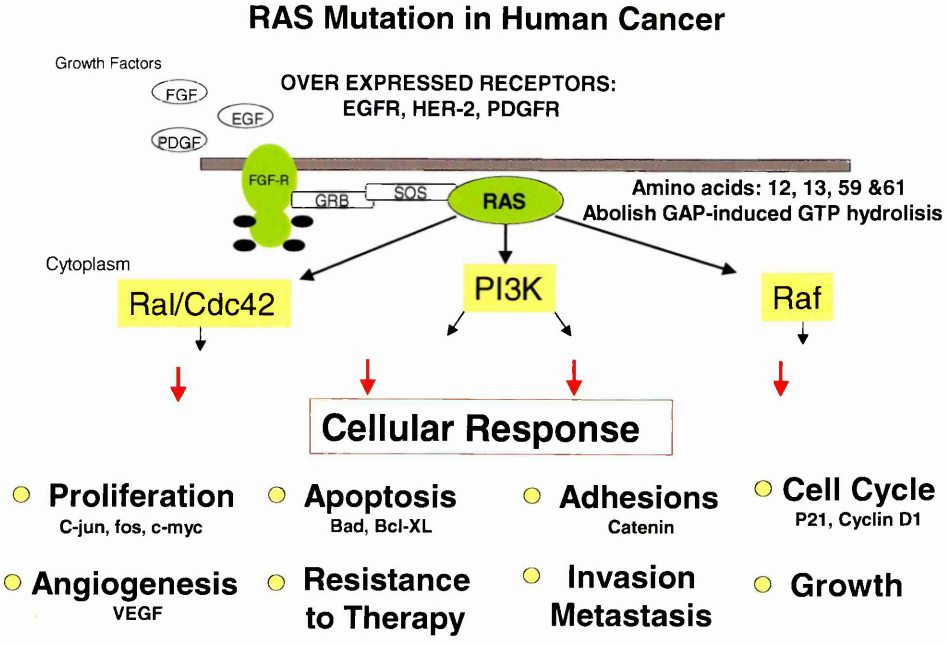


Figure 1.10 Ras signaling in human cancer.

The two downstream pathways targeted by the activated Ras that have been extensively linked to tumorigenesis are: (i) the Raf/MEK/ERK pathway and (ii) the PI3-kinase pathway. Mutation in Ras or aberrant signal transduction from the overexpressed receptor tyrosine kinases or G protein-coupled receptors in the surface of the tumor cells are the primary source for the oncogenic activation of Ras proteins. Once activated, Ras targets Raf in the plasma membrane, where Raf is activated and than phosphorylates the duel-specific kinases MEK 1 and 2. MEK then activates ERK 1 and 2 by phosphorylation. The activated ERK translocates into the nucleus and it interacts with different transcription factors and induces gene expression that subsequently promotes cell growth, differentiation and apoptosis. Uncontrolled Raf/MEK/ERK signaling has been observed in several tumors. The second downstream signaling cascade of Ras is PI3-K, which is extremely important for translating signals from a variety of extracellular stimuli into different cellular responses. PI3-K controls the phosphoinositide lipid metabolism and production of PIP_3 at the plasma membrane, which is involved in the recruitment and activation of a wide variety of downstream targets, including serine/threonine kinase Akt/PKB. The main effects of aberrant activation of the PI3-K/Akt pathway in the process of transformation are the support of cell survival and cell proliferation.

1.3.9 Ras signaling and COX-2 expressing in the tumor

Both the transcriptional and post-transcriptional mechanisms are involved in Ras-mediated COX-2 induction during carcinogenesis. The transcription of COX-2 gene has been reported to be induced by the activation of ERK, p38 MAPK, Rho and JNK pathways, whereas the mRNA stability of COX-2 is increased through the activation of ERK, p38 MAPK and Akt/PKB signaling pathways, which known to contribute in the aberrant

induction of COX-2 synergesticelly in cancer. The post-trancriptional mechanisms that mainly located downstream of the Ras also maintain a cross talk with the Ras signaling cascade. Recent work in the Ras-transformed intestinal epithelial cells has demonstrated that TGF-β, a signaling pathway required for Ras-mediated invasion and metastasis, also synergistically enhances the COX-2 mRNA stabilization and COX-2 expression (Sheng et al., 2000). In the colorectal cancer cells, the constitutive activation of ERK pathway results in an elevated expression of COX-2. The inhibition of the ERK pathway reduces the COX-2 mRNA stability and the COX-2 induction in these cell lines (Dixon et al., 2000; Sheng et al., 2000). In the Ras-transformed intestinal epithelial cells, the induction of both the COX-2 promoter and COX-2 mRNA stabilization is depended on the ERK activation (Sheng et al., 1998b). The Ras induced sequential activation of the PI3-K/PDK/AKT/PKB by various growth factor receptors or mutated Ras signaling facilitates a number of cellular events associated with the cellular transformation. Constitutive activation of the Akt/PKB pathway

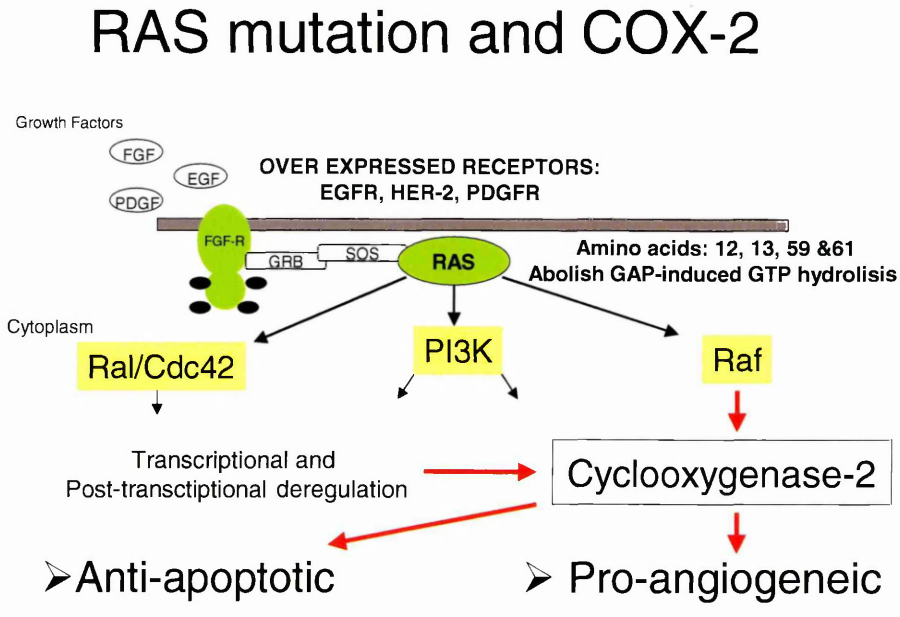
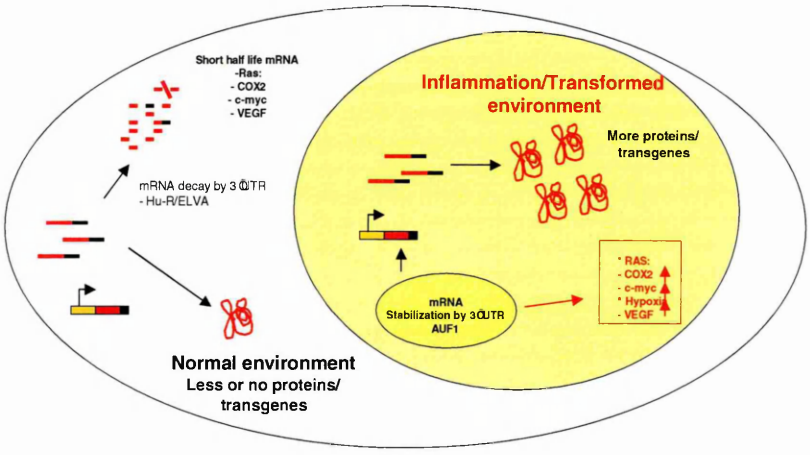


Figure 1.11 Mechanisms of COX-1 upregulation by Ras mutation in human cancer.

has been reported in colon cancer cells, which is associated with the COX-2 overexpression by modulating COX-2 mRNA stability (Sheng et al., 2000). The activation of this pathway is also required for the Ras-mediated stabilization of the COX-2 mRNA in the intestinal epithelial cells (Sheng et al., 2000).Activation of p38 MAPK works as a sensor for different cellular stresses and inflammations. Many inflammatory genes have been found to be regulated by p38 MAPK both transcriptionally and post-trancriptionally. A number of reports demonstrated the

ability of pro-inflammatory signals such as p38 MAPK to induce different pro-inflammatory genes expression by promoting a stabilization of different ARE-containing mRNAs including COX-2 mRNA. In the LPS-treated human monocytes, the p38 MAPK activation is required for selective COX-2 mRNA stabilization (Dean et al., 1999 #281). The association between chronic inflammations with cancer development is well documented. Constitutive activation of pro-inflammatory signaling cascades such as p38 MAPK has been observed in neoplastic tissues (Hardwick et al., 2001). In both intestinal and breast cancer cells, the aberrant activity of p38 MAPK promotes a COX-2 stabilization (Sheng et al., 2000). The anti-inflammatory drug dexamethasone abolishes p38 MAPK activity by inducing the expression of mitogen-activated protein kinase phosphatase 1 (MKP-1), which inhibits the mRNA stabilizing functions of p38 MAPK and decreases COX-2 expression (Lasa et al., 2001).

1.4 Hypothesis



Over-expression of various proteins associated with rapid responses to inflammation and/or proliferation can be controlled at the level of mRNA stability. Since tumor cells continually recapitulate intracellular programmed of proliferation, we propose that tumor cell selective stabilization of mRNA can be used as a novel mean to control therapeutic gene expression in cancer gene therapy.

Chapter 2

MATERIALS AND METHODS

2.1 CELL BIOLOGY

2.1.1 Eukaryotic cell culture – General procedures

All manipulations involving cell culture were carried out in a sterile environment provided by a laminar flow hood. All tissue culture reagents were filter sterilized by passage through a 0.22 μm filter and stored in sterile autoclaved containers.

The cell lines used in this work were:

293 (Graham et al., 1977)

293A (Quantum Biotechnologies, qbiogene)

293T (Klages et al., 2000)

Human melanoma cell lines, a kind gift from Professor Hart, London)

Mel 624

Mel 888

A378M

RIE-iRas cell line with an inducible activated Ha-Ras^{Val12} cDNA was generated by using the LacSwitch eukaryotic expression system (Stratagene, La Jolla, CA) and was maintained in DMEM containing 400 μg/ml G418 (Life Technologies,Inc), 150 μg/ml hygromycin B (Invitrogen, Carlsbad, CA) and 10% FBS. A kind gift from Professor Beauchamp from Vandarbil Medical school.

The following human tumor cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% fetal bovine serum:

HT1080 human osteosarcoma cell line

HCT116 colorectal cancer cell line

LnCap, PC-3 prostate cancer cell line

BEAS-2B is an immortalized normal human bronchial epithelial cell line.

U118, U87, U251 gliomas

hTER: Primary human retina pigment epithelial cell hTER were purchased from Clonetech.

Adherent cell lines were grown as monolayers in plastic tissue culture flasks or dishes (Nunc, Nalge Nunc, Napeville, IL) in DMEM supplemented with 10% v/v heat-inactivated fetal calf serum (Gibco BRL, Life Technologies, Grand Island, NY) and incubated at 37 °C in 5 or 10% CO_2. Cells were grown until just subconfluent (approximately 2 to 4 days) and were subcultured 1:10, using trypsin (0.05% W/v)/5mM EDTA to detach the cells. Cell counts were performed using an Improved Neubauer haemocytometer and an inverted microscope (Olympus 1X70).

2.1.2 Storage and recovery of cells stored in liquid nitrogen

Cells were trypsinised, pelleted and resuspended at approximately 10^6 cells/ml in medium containing 10% v/v dimenthylsulphoxide (DMSO). 1 ml aliquots were transferred to 1.5ml Nunc cryotubes, which were then placed within a 1 °C Freezing Container (Nalgene) and stored in a 70°C freezer. Using this apparatus, the cells cooled at approximately 1 °C per minute. Frozen cells were then transferred to liquid nitrogen tanks (-196 °C) the following day.

Rapid thawing in a 37 ° C water bath performed recovery of cells from liquid nitrogen storage. Thawed cells were washed in 10ml of medium, harvested by centrifugation (110g for 5 minutes) and were then transferred to 25 cm^2 flasks containing fresh culture medium.

2.1.3 Gene transfer into eukaryotic cells

2.1.3.1 Growth selection system

I. Geneticin (G418 sulphate)

Geneticin is an aminoglycosied antibiotic related to Gentamicin and is toxic to both prokaryotic and eukaryotic cells. Introduction of the neomycin phosphotransferase gene into eukaryotic cells can confer resistance to Geneticin added to normal medium (Southern and Bert, 1982). Geneticin (Gibco, Life Technologies, Scotland) was added to DMEM to a concentration of 5 mg/ml for selective growth of B16 cells and to 1 mg/ml for other cell lines, these being the concentrations previously determined to be optimal for selective growth of these cells.

II. Puromycin

Purmycin inhibits protein synthesis in eukarotic cells by acting as an analogue of amunoacyl-tRNA thus causing premature chain termination. The purmycin-N-acetyl-transferase gene from *Streptomyces alboniger* may be expressed in mammalian cells and used as a selectable marker for purmycin resistance (Vara et al., 1986). For

2.1.3.2 Transfection protocols

2.1.3.2.1 Calcium phosphate/DNA co-precipitation (ProFection)

This method involves mixing DNA with $CaCl_2$ and a phosphate buffer to form a fine precipitate, which is deposited, onto the cultured cells. Reagents provided in a ProFection kit (promega) were used. Twenty-four hours prior to transfection, $5X10^5$ cells were plated out in a $25cm^2$ flask.10 µg of the plasmid DNA to be transfected were made up to 263 µl using sterile distilled water followed by the addition of 37 µl of 2M $CaCl_2$. 300 µl of 2x

HEPES (N-(2-hydroxyethyl)piperazine- N'- (2-ethanesulphonic acid)) buffered saline (supplied in the kit) was then added drop wise to the mixture, during which time a fine precipitate became visible. The sample was incubated at room temperature for 30 minutes and then added drop wise to the medium in the cell culture flask. On the following day the medium was removed and replaced with fresh medium.

2.1.3.2.2 Effectine Transfection

This method involves complexing DNA with a non-liposomal lipid and was performed as recommended by the manufacturer's guidelines (Qiagen). Briefly cells were prepared as for the calcium phosphate protocol. For the transfection of a $25cm^2$ flask 1 µg of DNA is first mixed with buffer EC to a total volume of 150 l. Next 8 µl of Enhancer was added to condense the DNA before mixing with 10 µl of Effectene. After standing for 5 minutes at room temperature 800 µl of media was added and the mixture added to the cells.

If the aim was to obtain stable transfectants, the cells were split into selection medium after another 48 hrs. One method was to serially dilute the cells in selection medium and plate them in 96 well plates. After about 10-14 days wells containing a single colony were identified and transferred to a 24 well then $25cm^2$ flask. The other method used was to plate cells in selection medium into 6cm dishes. After about 10-14 days resistant colonies were either pooled or individually lifted using trypsin-soaked filter paper microsquares and transferred to individual wells of 24-well plate, followed by expansion into larger cell culture flasks.

Transfections and Luciferase Reporter assays: Plasmids used in DNA transfections were purified by DNA maxi kit (QIAGEN, Valencia, CA). DNA concentrations were determined by UV spectrophotometers and confirmed by analytical agarose gel

electrophoresis. For transient transfection, cells were seeded and grown to 50-80% confluent for 24 h prior to transfection. For 6 well plates, cells were transfected with 1 μg of DNA per well with Lipofectin (QIAGEN, Valencia, CA) at a 2:1 to 4:1 Lipofectin:DNA mass ratio (depending on the cell type). Twenty-four hours after transfection, cells were washed with PBS and lysed in the lysis buffer provided with the luciferase kit (Promega, Madison, WI). The luciferase activity of pGL3-control plasmid in each cell line was considered as 100%.

2.1.4 ASSAYS

2.1.4.1 Cell survival assay

293 cells were plated at a density of $5X10^4$ cell/well of a 5 well plate. After overnight incubation they were transiently transfected using the ProFection protocol detailed above. After 24 hours the cells were washed. Those wells containing cells tranfected with suicide genes were then incubated in media containing the appropriate prodrug: for HSVtk this was 5 μg/ml ganciclovir, for CD this was 3 μmM 5-fluorocytosine. All other cells were incubated in normal media. 5 days after transfection-surviving cells were determined using trypan blue exclusion: cells were washed, trypsinised and collected in 1ml of media. 15 μl was then mixed with ~1 μl of trypan blue and the number of viable cells counted using an Improved Neubauer haemocytometer and an inverted microscope (Olympus 1X70). Counts were performed 2 times per sample.

2.1.4.2 MTT assay

To evaluate the selectivity of the cytophatic effect of the conditionally replicating adenovirus, 500-1000 cells were seeded in 96-well plates. On the next day cells were infected with AdDNMT or WT Ad5 at 1.0 MOI. At indicated times post infection, 200 μl

of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Roche, Indianapolis, IN) in cell culture media (2mg/ml) was added to each well. After 4 h incubation at 37°C, the precipitate was dissolved with 200 µl of dissolving solution provided by the manufacture (Roche, Indianapolis, IN). Plates were than read on a microplate reader at 540 nm, All assay were performed triplicate and plotted as percent of non-infected cells treated with MTT in the same condition.

2.1.4.3 GM-CSF ELISA

ELISA plates (Rainin) were first prepared with the addition of 100 µl capture antibody/well (R&D Systems anti-human GM-CSF antibody MAB615) at 2 µg/ml in PBS. The plate was then sealed and incubated overnight at RT. The plate was then aspired and washed three times with wash buffer(0.05% Tween 20 in PBS) using a MultiWash Plus microplate washer. The plate was then blocked with 300 µl of PBS containing 1% BSA, 5% sucrose and incubated at RT for 60 mins. The aspiration / wash procedure was repeated as described above and 100 µl/well of test sample and standards were added in triplicate and incubated for 2 hours at RT. Standards were made up from a stock of 118 ng/ml recombinant human CM-CSF (R&D Systems): 20 µl of stock was added to 2.36mls of diluent (0.1% BSA, 0.05% Tween 20 in PBS) giving an upper standard value of 1ng/ml. serial dilutions of 1:2 were then performed down to 15.625pg/ml.

The aspiration / wash procedure was repeated as described. The 'detection' Biotinylated anti-human GM-CFS antibody (R&D Systems antibody BAM215) was then added as 100µl to each well from a working stock of 1 µg/ml in diluent (0.1% BSA, 0.05% Tween 20 PBS). This was sealed and incubated for 2 hours at RT.

The aspiration / wash procedure was repeated as described. Streptaviding-Horseradish peroxidase (Zymed laboratories Inc., San Francisco, CA) was then added as 100 µl to each well: 1:5000 in diluent (0.05% Tween at 20 in PBS) of a 1.25mg/ml stock. This was sealed and incubated for 20 minutes at RT.

The aspiration / wash procedure was repeated as described. 100 µl of 'substrate' solution (1:1 mixture of H_2O_2 and Tetramethylbensidine (BD PHamingen, San Diego, CA)) was then added.

2.1.5 Flow cytometry for cell cycle analysis

For cell cycle analysis, cell were collected by trypsinizing, washed twice with PBS, fixed with 95% ethanol and stored at 4°C for 24 h. These cells were treated with Rnase A (Roche, Indianapolis, IN), and were stained with propidium iodide (PI) (Sigma) at the final concentration of 25 µg/ml. The PI fluorescence of nuclei was measured using FACScane with FL2.

2.1.6 Quantitative analysis of mRNA by Northern blot

Total cellular mRNA was extracted by using an Rneasy kit (QIAGEN, Valencia, CA), according to the manufacture's protocol. The mRNA samples (5-10 µg/lane) were separated on formaldehyde-agarose gels and blotted onto nitrocellulose membranes. Next the RNA concentration was estimated by absorbance at 260 nm as previously described. 10 µg of total RNA was made up to 20 µl with diethyl pyrocarbonate (DEPC) treated distilled water for each sample. 2.5 µl of 5x RNA loading buffer (64 µl 5% bromophenol blue, 80 µl 0.5 M EDTA, 720 µl 37% formaldehyde, 2 ml glycerol, 3.084 ml fromamide, 4ml 10x MOPS, made up to 10ml with DEPC dH_2O) was added. (10x MOPS is 200mM 3-[N-morhilino] propanesulfonic acid (MOPS), 50mM sodium acetate, 10mM EDTA). The samples were

then heated to 65 °C for 4 minutes and kept on ice before loading on to a 1.2% agarose gel (1.6g agarose, 15ml 10x MOPS, made up to 150 ml with DEPC dH_2O was heated to fully dissolve the agarose. After cooling to ~ 65 °C 2.7 ml formaldehyde and 5 µl ethidium bromide was added and the gel poured). The gel was then equilibrated by running for 30 minutes at 80V in 1x running buffer (100ml 10x MOPS, 20 ml 37% formaldehyde, 880 mo DEPC dH_2O). after equilibration the samples were loaded on to the gel and run at ~80V for ~2 hours.

Northern blot of luciferase mRNA: The blots were hybridized with the 1.7-kb HindIII/XhoI fragment from pGL3 control containing the full length luciferase ORF cDNA probes labeled with [α-^{32}P] dCTP by random primer extension (Stratagene, La Jolla, CA) (Mawji et al). After hybridization and wash, the blot was subjected to autoradiograph. 18S rRNA signals were used to determine integrity of RNA and equality of the loading. For determination of mRNA stability, cells were transfected with the1µg of pGL3 control or pGL3 DNMT 3'UTR plasmids. 12-16 h post transfection the transcription was stopped by the addition of 10 µg/ml Act D (Sigma). The RNA samples were isolated at 0, 3, 6, 9 and 12 hour following the Act D treatment and analyzed for mRNA levels by Northern blotting.

Northern blot of E1A mRNA: For determination of mRNA stability, infected (10 moi of Ad-E1A-COX) and transfected (1µg of CMV-E1A or CMV-E1A-COX plasmids) RIE-iRas cells were treated with or without IPTG for 24h, then the transcription was stopped by the addition of 100µM DRB (5,6-dichlorobenzimidazole riboside; Sigma). The RNA samples were isolated at 0, 1, 2, 3, 4, and 5 hour following the DRB treatment and analyzed for mRNA levels by Northern blotting.

2.1.6 Quantitative analysis of protein by western blot

Protein extracts were prepared at 11-14 h postinfection by lysis of infected cells with radioimmunoprecipitation assay buffer (10mM Tris buffer [pH7.4], 425mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 100µl of protease inhibitor cocktail [Roche, Indianapolis, IN], 5 mM EGTA, 100 µM Na_3VO_4, 50 mM NaPyrophosphate, 50 mM NaF) and protein expression was determined by Western Blot after separation of 10µg of cell lysate on 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). The protein concentration was determined using a BCA protein assay with bovine serum albumin as a standard (Pierce, Rockford, IL).

The detection of adenovirus E1A and H-RAS proteins were accomplished using rabbit polyclonal antibody (Santa Cruz Biotech., Santa Cruz, CA) against the target proteins. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL). The detection of adenovirus fiber protein, a monoclonal antibody from NeoMarker was used. For the detection of Phospho-MAP kinase a monoclonal antibody from New England Biolabs Inc. was used. The MAP kinase specific inhibitor PD98059 was also purchased from New England Biolabs Inc.

2.1.7 Preparation of complementary DNA for analysis with PCR

The RNA sample was first incubated with 1 µl DNAse (RNAse Free) (Boerhinger Mannheim) and incubated at 37 °C for one hour. Next the RNA concentration was estimated by absorbance at 260 mm as previously described. A First strand cDNA was generated from an RNA template using a First Strand cDNA Synthesis Kit supplied by Boerhinger Mannheim Roche (Indianapolis, IN). For each RNA sample two aqueous solutions containing 1 µg of total RNA were made up to 10 µl with sterile water. To one

sample 10 µl of the 'Reaction Mixture' containing RNAase inhibitor, magnesium chloride, DNTPs, aqueous buffer and 2 µl Oligo-p(dT) $_{15}$ primer was added; this was the rt negative control. To the other sample was added the same reaction mixture plus 1 µl AMV reverse transcriptase; this was the rt positive sample. All samples were then incubated at 25 °C for 10 minutes and then at 42 °C for 60 min. For analysis with polymerase chain reaction (rtPCR) 2 µl of the reaction mixture was used in each PCR sample. Both rt positive and negative samples were first analyzed for glyceraldehydes phosphate dehydrogenace (GAPDH) to confirm a lack of DNA contamination of the mRNA and identify equal quantities of input RNA to the rtPCR procedure. The GAPDH primers used were from the human GAPGH PCR primer pair (R&D system, Minneapolis, MN) with the following sequence:

Forward: AAAGGGTCATCATCTCTGCC

Reverse: TGACAAAGTGGTCGTTGAGG

PCR was performed in a 50µl reaction mixture with 250µM of each dNTP, 100nM of primers, 5µl of 10x buffer (HT Biotechnology Ltd, Cambridge, U.K.) and 1 unit of super Taq DNA polymerase (HT Biotechnology Ltd, Cambridge, U.K.) using 30 cycles (94°C, 1 minute denaturation; 58°C, 1.5 minute annealing; and 72°C 2 minutes extension). For GAPDH positive PCR is identified by a band at 576 base pairs. Subsequent analysis by PRC of the rt samples was performed using primers of interest with the appropriate PCR conditions.

The reaction mix (25µl samples) was analyzed by agarose gel electrophoresis (1%) in TAE buffer containing 0.2µg/ml ethidium bromide. In all experiments, a mock PCR (without added DNA) was performed to exclude contamination. To exclude carry over of genomic

DNA during the RNA preparation step, controls were also carried out in which the reverse transcriptase enzyme was omitted.

2.2 MOLECULAR BIOLOGY

2.2.1 General Procedures

Distilled water was used to prepare all the solutions utilized to prepare and manipulate nucleic acids. The solutions were stored in a sterile container after being either autoclaved before use or filter sterilized when thermolabile. Sigma (St.Louis, MO) supplied all chemical reagents and New England BioLabs (Beverly, MA) supplied all the enzymes, unless differently stated.

2.2.2 Determination of nucleic acid concentration

When measuring the absorbance of an aqueous solution of the nucleic acid at 260nm, it was determined that a double standard DNA concentration of 50 μg/ml is equivalent to an absorbance on one unit while 40 μg/ml represented an RNA concentration.

2.2.3 Amplification of DNA sequences by the polymerase chain reaction

Two types of *Thermus aquaticus* (*Taq*) DNA polymerase (AmpliTaq for diagnostic purpose or AmpligTaq for cloning purposes) were used to perform polymerase chain reactions (PCR) when samples containing template DNA mixed with sequence-specific oligonucleotide primers were cycled through three temperature incubations: 1. Denaturation of double stranded DNA; 2. Annealing of primers to DNA 3. Extension of target sequences by *Taq* DNA polymerase.

The PCR was carried out in a Biometra TRIO-thermoblock (Biometra, Gottingen, FRG)

The optimal cycle number and exact annealing and extensions were as described for each individual reaction (see Results). Primers were synthesized by the Molecular biology Core Facility, Mayo Foundation , on an Applied Biosystems 380B Synthesizer.

The reaction mixtures were prepared in a laminar flow hood isolated from normal areas of DNA handling. Each reaction sample consisted of template DN (1 μg of genomic DNA or 0.1-0.5μg of plasmid DNA; for semi-quantitative rtPCR the cDNA equivalent of 0.1 μg RNA was used), 8 μl dNTPs (40 mM), 5 μl of 10x PCR buffer, 0.2 μg 5' primer, 0.2μg 3'promer, 0.5 μl *Taq* DNA polymerase (5 units/μl) and distilled water added to a total volume of 50 μl. The reaction was then heated to 94°C for 10 minutes and then allowed to proceed through 20 to 30 cycles of denaturation, annealing and extension to produce the required degree of amplification. If the PCR product was required for cloning experiment a final 10-minute extension cycle at 72°C was added. The amplified PCR products were evaluated by mixing 12 μl of the reaction mixture with 2 μl of the reaction mixture with 2 μl of 6x loading buffer stock solution and run on an agarose gel.

2.2.4 Ligation of PCR products

PCR products were ligated into the pCR3.1 vector using a TA Cloning Kit (invitrogen, Carlsbad, CA). This system takes advantage of the nontemplate-dependent activity of Taq polymerase that adds a single deoxyadenoisine to the 3' termini of the double stranded molecules. The linearised vectors that are supplied possess single overhanging deoxythimidine residues at the 3' termini, thus allowing the PCR product to ligate efficiently with the vector. The ligation reactions were performed according to the manufacturer's instructions in 10 μl volumes consisting of : 1 μl of 10x ligation buffer, 1μl T4 DNA ligase, 2 μl linearised vector (60 ng pCR3.1), 1 μl PCR reaction mixture and 5 μl

distilled water. The reaction mixture was incubated overnight at 14°C and was then transformed into competent e.coli (TOP10F' strain for pCR3.1) and plated onto L-agar containing kanamycin.

2.2.5 Agarose gel electropheresis of DNA

Gels were prepared by adding agarose (0.7 to 1.8% w/v) to 150ml x TAE (Tris-acetate-EDTA) buffer (diluted from 50X TAE stock solution: 2M Tris base, 2M glacial acetic acid, 50 mM EDTA) and boiled in microwave cooker for r5 minutes. On cooling to below 50°C, 2 µl of ethidium bromide stock solution (10 mg/ml) was added. Gels were poured into a gel former with a well-comb in place. After setting, the gel was submerged in an electropheresis tank containing 1 x TAE buffer. Loading buffer (1/6 volume of 6x stock solution: 0.25% bromophenol blue, 40% w/v sucrose in water) was added to the DNA solutions, which were then transferred into the wells, and electropheresis was performed using a voltage between 70 and 110 volts. Geh gel was transilluminted with short wave ultraviolet lights and the DNA was visualized by 2uv transilluminator (UVP, Upland, CA) and Alpha Ease 5.04 Software (Alpha Innotech Corporation, San Leandro, CA), DNA fragment were sized by reference to a 'DNA ladder'.

2.2.6 Transformation of bacteria

The plasmid DNA was added to 100 µl of competent *E.coli.* The suspension was cooled on ice for 45 minutes, warmed at 42 °C for 1 minute and then returned to ice for 2 minutes. 400 µl of L-broth was then added to the samples followed by incubation in a shaking incubator at 37 °C for 1 h to permit expression of the antibiotic resistance gene on the plasmid. The bacteria were then plated out onto 90mm Petri dishes (Becton Dickenson Labware, NJ) containing L-agar (L-broth with 1.5% w/v agar) with ampicillin (final

concentration of 100 µl/ml) or Kanamycin (final concentration of 25 µl/ml). The plates were incubated overnight at 37°C.

2.2.7 Small scale preparation of plasmid DNA ("miniprep")

Plasmid DNA was prepared from small cultures of bacteria using a QIAprep 8 plasmid minipreparation kit and QIAvac Manifold 6S (Qiagen, Valencia, CA), following the protocol supplied by the manufacturer. This procedure was based on the alkaline lysis method for rapid extraction of plasmid DNA from bacterial cells followed by the absorption of DNA onto silica in the presence of high salt.

Single bacterial colonies were inoculated into 5ml of L-broth containing ampicillin and incubated overnight in a shaking incubator at 37 °C. 1.4ml of the overnight cultures were centrifuged at 10,000g for 5 minutes and bacteria were then resuspended in 250 µl of resuspension buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA, 100 µg/ml RNAse). 250 µl of lysis buffer P2 (200mM NaOH, 1% SDS) was then added and mixed, followed by adding 500 µl of neutralization buffer N3 which adjusts the sample to high salt binding conditions and causes precipitation of denatured proteins, SDS, cellular debris and chromosomal DNA. The samples were then centrifuged at 10,000g for 10 minutes and the supernatants were then transferred to individual wells of a QIAprep 8 strip placed in a QIAvac Manifold 6S. Vacuum suction was applied to cause flow through the silica membrane, which forms the floor of the wells. After washing with 2 ml of buffer PE to remove salts, the DNA was eluted by applying 100 µl of distilled water to the silica membrane.

2.2.8 Large scale preparation of plasmid DNA ("maxiprep")

Qiagen Plasmid Maxi kit was used which is based on the modified alkaline procedure followed by binding of plasmid DNA to an anion-exchange resin. A single bacterial colony was used to inoculate a 2 ml volume of L-broth containing ampicillin which was incubated for 8h in a shaking incubator at 37°C. 1ml of this culture was used to inoculate 100 ml of L-broth containing ampicillin which was then incubated overnight. The bacteria was pelleted by centrifugation at 6,000g for 20 minutes (J2-HS centrifuge, Beckman)and resuspended in 10 ml of resuspension buffer P1. 10ml of lysis buffer P2 was then added and left at room temperature for 5 minutes. 10 ml of neutralization buffer P3 (3M potassium acetate pH 5.5) (pre-chilled to 4°C) was added and the lysate poured into a QIAfilter Maxi cartridge and incubated at room temperature for 10 minutes. The cell lysate was then filtered onto a QIAGEN-tip which had been pre-equilibrated with 10ml buffer QBT (750ml NaCl, 50mM MOPS pH 7.0, 15% ethanol, 0.15% Triton X-100) and allowed to enter the anion-exchange resin by gravity flow. Under these conditions, the plasmid DNA binds to the anion-exchange resin. The resin was then washed with 0 ml of medium salt buffer QC (1M NaCl, 50mM MOPS pH 7.0, 15% ethanol) to remove RNA, proteins and low molecular weight impurities. The DNA was eluted with 15ml of high salt buffer QF (1.25M NaCl, 50mM Tris-HCl pH 8.5, 15% ethanol), and was then desalted by precipitation with 10.5ml isopropanol. The DNA was pelleted by centrifugation at 15,000g for 30 minutes at 4°C, washed with 70% v/v ethanol, air dried and then dissolved in TE buffer.

2.2.9 Digestion of DNA with restriction enzymes

Plasmid DNA was digested in volumes of 30 µl using 1-2 units of enzymes per µg of DNA, buffers supplied by the manufacturer and incubated for 60 minutes at the appreciate temperature; BSA was added when indicated.

2.2.10 Removal of 5' terminal phosphate groups

To reduce re-ligation of the vector DNA in cases where cohesive ends were present, treatment with calf intestinal alkaline phophatase (CIAP) to remove the 5' phosphate groups of linear double stranded DNA was performed. At the end of restriction enzyme digestion, 1 unit of CIAP (Promega, Madison, WI) was added to the reaction sample with 5µl of 10x reaction buffer (50mM Tris-HCl pH 9.3, 1mM $MgCl_2$ and 1mM spermidine) and the reaction mixture made up to 50 µl with DH_2O. This was then incubated for a further 60 minutes at 37°C. the sample was then run on an agarose gel and the appropriate fragment was purified as described above.

2.2.11 Purification of DNA restriction fragments

Agarose gels were visualized by UV transillumination and the bands of interest excised using a scalper blade. The DNA was purified from the gel using the QIAquick gel extraction kit (Qiagen, Valencia, CA) following the instructions provided by the manufacturer. The method is based on the binding of DNA to silica under high salt conditions. The excised portion of gel was dissolved in 3 volumes of buffer QG and incubated at 50 °C for 10 minutes. Once the gel had completely dissolved 1 volume of isopoppanol was added if the DNA fragment was between 500-4000 base pairs. The sample was then added to the QIAquick column and centrifuged at ≥10,000g for 1 minute. The column was then washed with 500 µl of buffer QG and centrifuged as before. 750 µl of buffer PE was then added and centrifuged as before. The DNA was elute form the

column by the addition of 30 µl TE, waiting 1 minute before recentrifugation, 1 µl of the elute was run on an agarose gel to confirm successful purification of the DNA fragment.

2.2.12 Ligation of DNA fragments into vectors

Ligations were performed overnight at 14 °C in volumes of 15 µl using 1 unit of T4 DNAligase and ligase buffer (50mM Tris-HCl pH 7.8, 10 mM $MgCl_2$ 10mM DTT, 1mM ATP, 25µg/ml BSA). Reaction samples were such that the concentration of the 5' temrinin was 0.1-1.0 µm. The molar ration of vector to insert was in the range of 1:3 to 1:10.

2.2.13 Plasmid construction

The 469bp human COX-2 3'UTR cDNA clone was isolated by reverse transcription-polymerase chain reaction (PCR) amplification using human COX-2 sequence-specific primers. PCR products were legated into the TOPO TA-cloning vector (Invitrogen, Carlsbad, CA) and subsequently excised with *Xho*1. The DNA fragments were purified by agarose gel electrophoresis and extracted using Gene clean Kit (QIAGEN, Valencia, CA). DNA inserts were ligated into the unique *Xho*1 site of the pE1A-K2 vector (pE1A-K2-COX), located in the 3'-end of the adenovirus type 5 E1A gene.

The pCR3.1-GALV expression plasmid consists of the human CMV promoter driving expression of the hyperfusogenic Gibbon Ape Leukemia Virus (GALV) envelope cDNA as described in {Fielding, 2000 #2372} and {Bateman, 2000 #2629}. The 469bp human COX-2 3'UTR cDNA clone was isolated by reverse transcription-polymerase chain reaction (PCR) amplification using human COX-2 sequence-specific primers.

The plasmid pGL3-control was purchased from Promaga. For the construction of pGL-3 DNMT 3'UTR, human DNMT-3' UTR (5090-5408) was amplified by reverse transcription (RT) –PCR from 1µg of total RNA prepared from human melanoma Mel624 cell line. Reverse

transcription was carried out with random primers (Roche Molecular Biochemicals), AMV Reverse transcriptase (Roche Molecular Biochemicals) and 1.0 μg of total RNA as recommended by the manufacturer in a total volume of 20 μl. Two micro liter of reverse transcribed cDNA was used for subsequent PCR amplification of the 3'UTR with *Taq* polymerase (Roche), 50 ng of the sense primer (5'-CTCGAGTCTGC CCTCCCGTCACCC-3') and antisense primer (5'-CTCGAGGGTTT ATAGGAGAGATTT-3'), 1 mM dNTPs, and the manufacturer's amplification buffer. Cycling conditions were as followed: 95°C for 1 min, 53.9°C for 1.5 min, and 72°C for 1.5 min for 30 cycles. The amplified fragment was subcloned into pCR2.1 TOPO using the TA cloning kit (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. To generate pGL3 DNMT1 3'UTR, the 318 bp 3'UTR was excised from pCR2.1 TOPO DNMT 3'UTR with Xba1 and Spe1 and legated into the Xba1 digested pGL3-control (Promega, Madison, WI) vector.

2.3 Construction and production of recombinant adenovirus

2.3.1 Ad-E1A-COX virus

The 469bp human COX-2 3'UTR was cloned as described in the previous chapter. The Ad-E1A-COX is an *E1/E3* deleted, serotype 5 vector that contains the cytomegalovirus (CMV) immediate-early gene promoter-enhancer driving the adenovirus E1A cDNA (1100bp) which is fused with COX-2 3' UTR (469bp). This vector was constructed by using an AdEasy kit, according to the manufacturer's protocol (Qbiogene,CA). Briefly, E1A-COX-2 gene was PCR cloned from plasmid pE1A-K2-COX and inserted into the transfer plasmid, pShuttle (AdEasy kit, Qbiogene,CA) by using the unique *Hind*III-*Eco*RV sites. The resulting plasmid (pShuttle-E1A-COX) was than linearized with *Pme*1 and co-transfected into *E.coli* strain BJ5183 together with pAdEasy-1 (Qbiogene,CA), the viral DNA plasmid. The recombinant adenoviral

construct was then cleaved with *Pac*I to expose its Inverted Terminal Repeats and transfected into 293A cells to produce viral particles. The viral clones were screened by PCR diagnosis of Hirt extracts. The selective vector clone was then plaque purified at least three times before it was used in experiments. For *in vivo* experiments, the virus was purified on cesium chloride gradient columns.

2.3.2 AdDNMT virus

The novel conditionally replicating adenovirus was constructed using the Microbix (Hamilton, Canada) system, according to the manufacture's protocol (Qbiogene,CA). Briefly, the 1498bp adenovirus-5 E1A transcriptional unit with the E1A promoter was PCR amplified from the Adenovirus-5 genomic DNA (Sigma) using 50 ng of sense primer (5'-CATCATAATATACCTTATTTTGG-3') and antisense primer (5'-GCTAGCCCATGAGGTCAG ATGTAACCAAGA-3'). The PCR product than gene cleaned (QIAGEN, Valencia, CA) and subcloned into pCR2.1 TOPO vector. After the sequence was verified, the E1A transcriptional unit was excised as a NotI/EcoRV fragment and legated into the shuttle vector pDC512, which also contain an XbaI/HindIII fragment of DNMT1 3'-UTR upstream of the SV40 polyA (p512-E1A-DMNT3'). The control shuttle vector do not contain any DNMT1 3'-UTR (p512-E1A). Recombinant viruses (AdDNMT and Ad E1A) were generated by site directed homologous recombination in 293 cells followed by calcium phosphate transfection of p512-E1A or p512-E1A-DMNT3' with the adenoviral genome contain in pBGHfrt (Microbix, Hamilton, Canada). Individual plaques were expended in 293 cells and purified using cesium chloride gradient ultracentrifugation (McGrory, 1988 #18). Viral titers were determined by the plaque-

forming assay in 293 cells and expressed as pfu/volume. The viral titer range from 10^{10} to 10^{11} pfu/ml. Wild type adenovirus was purchased from Microbix (Hamilton, Canada).

2.3.3 Wild type replication competent adenovirus

Wild type adenovirus serotype 5 was purchased from Qbiogene, CA. The virus was grown on 293 cells and purified on cesium chloride gradient columns for use in *in vitro* and *in vivo* experiments.

2.4 In vivo studies

To establish subcutaneous tumors, 4-5 week old athymic nu/nu female mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with $2X10^6$ tumor cells (U118, U87, U251). When the tumors measured 0.3 cm in diameter, $1X10^8$ pfu of wild type Ad-5 or Ad-E1A-COX were injected intratumorally in a 0.05 ml volume. Control tumors were injected with equal volume of PBS only. Animals were examined every other day and euthanized if tumor size reached 1.0X1.0 cm. An animal was scored as tumor-free when tumor size remained <0.2 cm.

For evaluation of the relative uptake of virus into liver after i.v. injection, athymic nu/nu female mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were given $1X10^8$ pfu of wild type Ad-5 or Ad-E1A-COX in single tail vein injection and euthanized at 24 and 72 hours postinoculation. Liver, blood and spleens were excised, divided, and processed for viral titer, western blot or RT-PCR. To determine the virus titers, tissues were weighed, homogenate and freeze/thawed three times, centrifuged (3000Xg), and the virus titer in the supernatant was determined by a plaque assay using 293A cells.

To establish subcutaneous tumors, 4-5 week old athymic nu/nu female mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) were injected with $2x10^6$ tumor cells (U118,

U251). When the tumors measured the appropriate size (palpable (0.2cm)) or 0.5-0.6cm, plasmid DNA, wild type Ad-5 or a combination was injected intratumorally in 50μl or 100μl volume. In order to administer the combination treatment intratumorally, the pGALV and Ad5-Wt components were mixed in a syringe and injected simultaneously. Animals were examined every other day and euthanized if tumor size reached 1.0x1.0 cm.

2.5 Biodistribution and liver toxicity studies

Several doses were evaluated to assess a maximum tolerated i.v. dose of Ad-E1A-COX in immunocompetent C57BL/6J (Jackson Laboratory, Bar Harbor, Maine) mice. Desired amount of virus was diluted in PBS and injected into the tail vain in a volume of 0.1 ml. Mice were weighed and observed daily for signs of acute toxicities (such as lethargy or anorexia). When signs of acute toxicity were observed, mice were euthanized. Surviving animals were euthanized 14 days after virus injection. Gross pathological changes were recorded at necropsy. Liver were collected from all the mice, half of the liver was preserved in formalin for histopathological analysis and the other half was weighted, flash-frozen in liquid nitrogen, and stored at -70°C. These liver samples were then weighed, homogenated and used for determining the virus titer and examined for the presence of the viral DNA by southern blot.

For the evaluation of the relative uptake of virus into all the major organs (liver, lung, heart and kidney) after i.v. injection, immunocompetent C57BL/6J (Jackson Laboratory, Bar Harbor, Maine) mice were given $5X10^9$ pfu of Ad-E1A-COX and wt-Ad5 virus in a single tail vain injection and euthanized at 24, 48 and 72 hours post inoculation (n=3 per time point). Serum was collected by cardiac bleeds and was analyzed for selective clinical

chemistry parameters including aspartate aminotransferase (AST) and alanine aminotransferase (ALT). All the organs were excise, divided and processed as described above for viral titer and southern blot for viral DNA.

Chapter 3

Cloning and *in vitro* characterization of the COX-2 3'untranstated region

3.1 Introduction:

Uncontrolled gene expression is a hallmark of cancer cells. Acquiring a new profile of gene expression helps cancer cells to overcome different cellular checkpoints and give them a growth advantage over normal cells. An efficient and rapid way to alter gene expression is via altering the stability of transcribed mRNAs. Many proto-oncogenes and proinflammatory cytokines require rapid and transient induction in response to extracellular stimuli, which involves both transcriptional and post-transcriptional mechanisms of gene expression. The half-life of these labile mRNAs is partly dependent upon specific cis-acting elements found in the 3' untranslated region (3'UTR). The cis-acting destabilizing elements can be variable in sequence and length, but are well characterized by AU-rich regions (ARE), which contain multiple copies of AUUUA repeats within the U-rich sequence (Shaw & Kamen, 1986). ARE-mediated mRNA stability and translation are controlled through a complex network of RNA/protein interactions involving recognition of ARE-containing specific target mRNAs by specific RNA-binding proteins (RNA-BPs) in the presence of the appropriate signals. Abnormal expression of RNA-BPs, mutation in the cis-acting regulatory sequence in the target mRNAs or modulation of signaling pathways are all known to alter mRNA stability and induce deregulated gene expression in cancer cells. These alterations occur in diverse cancer types, which result in a deregulation of many genes involved in cancer progression.

It has been reported that the ARE sequence from some cancer associated or proinflammatoty genes is sufficient to change a stable long-lived mRNA, such as β-globin mRNA to an unstable form (Shaw & Kamen, 1986; Sheng et al., 2001). But in the presence of appropriate stimuli such as oncogenic or proinflammatory signals, the same ARE is able

to reverse the destabilizing effects and to increase the reporter mRNA half-life significantly. In this chapter, we hypothize that we may be able to use the ARE sequence within the 3'UTR region of a tumor associated gene to stabilize therapeutic/viral mRNA within the tumor and thus achieve tumor selective expression of therapeutic/viral genes. We used the ARE from a well documented cancer associated gene cyclooxygenase 2 (COX-2) to show that an essential replicative viral gene can be expressed selectively in Ras activated transformed cells.

Many studies have linked elevated expression of COX-2, a key enzyme in prostaglandin synthesis, to the pathology of breast, colorectal, head and neck and other cancer types (reviewed in Turini & DuBois, 2002). Expression of COX-2 is normally induced in cells by cytokines, growth factors and tumor promoters (Turini & DuBois, 2002). Up-regulation of COX-2 is a downstream effect of RAS-mediated transformation (Sheng et al., 2001; Sheng et al., 1997b; Sheng et al., 1998b). Although RAS-mediated overexpression of COX-2 is also associated with an increased transcription of the COX-2 gene, a large component of its up-regulation is mediated by selective stabilization of the mRNA of the COX-2 gene in RAS-transformed cells (Sheng et al., 2000) (Dixon et al., 2000; Sheng et al., 2001) mRNA stability has been mapped to the 3'Untranslated Region of the COX-mRNA (Dixon et al., 2000) This mRNA stabilization was mediated in part through the activation of the mitogen activated protein kinase P-MAPK pathway, which is a well characterized downstream effectors of both RAS-, and EGF-receptor mediated intracellular signaling (Sheng et al., 2001) (Montero & Nagamine, 1999). Finally, the P-MAPK signaling cascade has previously been shown to be involved in preferential

stabilization of other growth promoting mRNAs (Brook et al., 2000) and proteins that link RAS-mediated oncogenesis.

In this chapter, we describe a novel mean to achieve tumor restricted gene expression by using COX-2 ARE-mediated tumor selective mRNA stabilization. We constructed the CMV promoter driven E1A-COX2 plasmid by inserting 469bp of the human COX-2 3'UTR cDNA in the 3'-end of the adenovirus type 5 E1A gene (**Figure 2**). By using the RIE-iRAS model cell system, we demonstrated that the CMV-E1A-COX2 construct could complement, *in trans,* the mobilization of a replication incompetent Ad-GFP adenoviral vector by expressing appreciable functional levels of E1A protein only in the presence of IPTG induced activated Ha-Ras^{Val12} protein. Moreover, using inhibition studies, we confirmed that COX-2 3'UTR-mediated stabilization of E1A expression in this system requires the activated P-MAPK signaling pathway, which is up-regulated in the presence of an activated Ha-Ras^{Val12}oncogene. Our results support the use of tumor selective mRNA stabilization mechanisms as a targeting strategy in the context of cancer gene therapy.

3.2 Results:

3.2.1 H-Ras mediated Conditional Cellular Transformation and COX-2 induction

It was previously reported that the increased expression of COX-2 during cellular transformation is a downstream effect of the aberrant activity of the RAS oncogene and a conserved AU-rich region located in the COX-2 3'UTR is required for RAS mediated COX-2 mRNA stabilization and tumor selective COX-2 induction (Sheng et al., 1997a) (Dixon et al., 2000). Based on these observations, we hypothesized that a therapeutic, or viral gene can preferentially be expressed by ligation to the COX-2 3'UTR to exploit tumor

cell selective mRNA stabilization. To test our hypothesis, we used an *in vitro* model system where rat intestinal epithelial cells (RIE-1) were stably transfected with an inducible Ha-Ras^{Val-12} cDNA and are referred to as RIE-iRas cells (Sheng et al., 1997a). In normal culture condition the RIE-iRas cells behave similarly to the parental RIE-1 cells and show nontransformed phenotypes, which include intact cell-cell contact inhibition (**Figure 1B-1** and (Sheng et al., 1997a). But in the presence of 5mM IPTG, an induction of the activated Ha-Ras^{Val-12} was observed by the western blot (Sheng et al., 1997a)(and **Figure 1A**). After 24-48 hours of treatment with IPTG, RIE-iRas cells acquire morphologic changes, such as a spindly appearance, growth in overlapping clusters and loss of contact inhibition (**Figure 1B-2**). The IPTG induced activated Ha-Ras^{Val-12} also gave the RIE-iRas cells significant growth advantages over the noninduced RIE-iRas cells (Fig. 1C) and the transformation could be completely reversed upon withdrawal of IPTG from the media for 48-72 hours (Sheng et al., 1997a and data not shown). In the Ha-Ras^{Val12} induced RIE-iRas cells, an elevation of COX-2 protein was detected by western blot 8-12 hours after the addition of IPTG and the half-life of the COX-mRNA was almost doubled (13 min. to 30 minute) (Sheng et al., 1997a). For all the future experiments, we used the RIE-iRas inducible system to evaluate our hypothesis.

3.2.2 E1A-COX complements adenoviral replication *in trans* only in the presence of activated RAS expression

To investigate effect of the COX-2 3'UTR on transgene expression, we inserted the 469 bp COX-2 3'UTR downstream of the adenoviral E1A gene, which is expressed under the CMV promoter (**Figure 2**). This construct was used to do the following vector mobilization experiment in the RIE-iRAS cells. Although RIE-iRAS cells are of rodent origin, they are

still able to support wild type adenoviral replication but at reduced levels compared to human cell lines (data not shown). RIE-iRAS cells transfected with CMV-E1A or CMV-E1A-COX were subsequently infected with an E1A-deleted replication incompetent adenoviral vector carrying a marker gene GFP. E1A-expressing cells would be converted into transient adenoviral producer cells if they subsequently become infected with the Ad-GFP construct and would, therefore, mobilize the GFP reporter gene through the cell monolayer. FACS analysis of transfected/infected RIE-iRAS cells demonstrated that CMV-E1A supported considerable mobilization of the incoming Ad-GFP vector irrespective of the presence of IPTG (**Figure 3A-3**). In contrast, CMV-E1A-COX was unable to mobilize the Ad-GFP vector to any significantly enhanced levels compared to mock transfected cells unless cells were previously induced with IPTG to express the Ha-Ras^{Val-12} oncogene (**Figure 3A-6 and 7**). To confirm that the mobilization of the GFP reporter gene was due to complementation *in trans* by the E1A proteins, supernatants were removed from the transfected/infected RIE-iRAS cultures and plated on HT1080 cells (**Figure 3B**). FACS analysis of the infected HT1080 cells indicated that similar titers of Ad-GFP were present in the supernatants removed from CMV-E1A/Ad-GFP treated RIE-iRAS cells irrespective of the induction of Ha-Ras^{Val12}; however, there was only a significant titer of Ad-GFP released from CMV-E1A-COX/Ad-GFP-transduced RIE-iRAS cells if these cells were treated with IPTG to induce expression of Ha-Ras^{Val-12} (**Figure 3B**). No cytopathic effect was observed in the infected HT1080 cells, indicating that the virus released from the RIE-iRAS cells was replication incompetent and derived from complementation of the Ad-GFP vector by the E1A proteins.

3.2.3 COX-2 3'UTR-mediated E1A stabilization in Ha-RAS^{val-12} transformed cells is dependent upon the MAP kinase pathway

Working downstream of RAS to mediate cellular transformation is the mitogen-activated protein kinase (MAPK) cascade. Activation of RAS by different growth factors and cytokines leads to phosphorylation and activation of the MAPK (P-MAPK) signaling cascade, which subsequently activate downstream different effectors molecules. Inhibition of the MAPK pathway had been reported to block RAS-mediated COX-2 mRNA stabilization and expression (Sheng et al., 2001; Sheng et al., 1998a). Therefore, we investigated whether the effects we observed with Ha-Ras^{Val-12}-mediated control of E1A-COX expression operate through the P-MAPK signaling pathway. RIE-iRAS cells express minimal levels of P-MAPK in the absence of Ha-Ras^{Val-12} induction (**Figure 4A**). However, the expression of the activated oncogene, even at relatively low levels, induces high levels of P-MAPK protein (**Figure 4A**). PD98059, an inhibitor of P-MAPK activity effectively blocked MAPK phosphorylation and activation in RIE-iRAS cells (Sheng et al., 1998a), even when the cells were induced to express high levels of Ha-Ras^{Val-12} (**Figure 4A**). Therefore, we used PD98059 inhibition to demonstrate that the E1A expression from CMV-E1A-COX is also dependent upon the P-MAPK pathway. Mobilization of the Ad-GFP adenoviral vector through RIE-iRAS cultures by the CMV-E1A construct was independent of IPTG induced Ras signaling (**Figure 4B-1 & 2**) and the MAPK inhibitor PD98059 did not have any effect on the mobilization (**Figure 4B-3**) (**Figure 4B**). As shown before, transfection of CMV-E1A-COX required IPTG induction of Ha-Ras^{Val-12} to be effective in vector mobilization (**Figure 4B-4**). However, inhibition of P-MAPK activity by PD98059 greatly reduced the ability of CMV-E1A-COX to support Ad-GFP

mobilization even in the presence of high levels of expression of Ha-Ras^{Val-12} induced by IPTG (**Figure 4B-5**). From the results of these experiments, we can conclude that RAS mediated MAPK activation is essential for the COX-2 3'UTR to stabilize the E1A gene and support viral replication.

3.2.4 COX-2 3' UTR able to regulate gene expression in different human tumor cell lines with elevated level of activated Ras/MAPK oncogenic signal

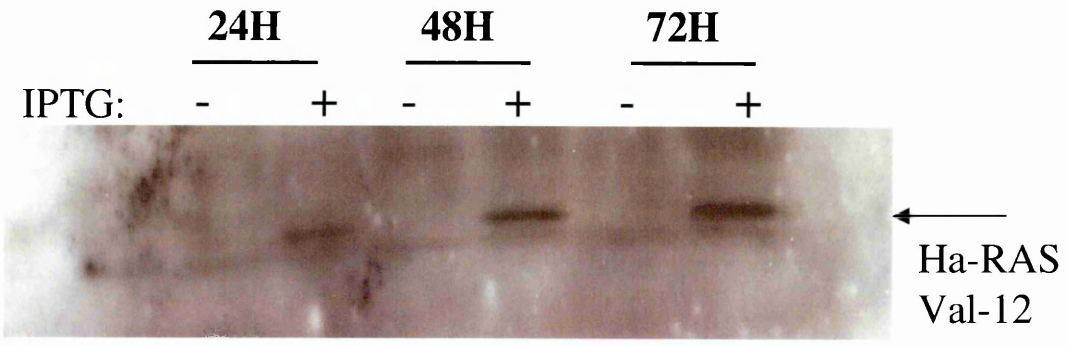
In our previous study we show that in an inducible model cell line, COX-2 3' UTR can stabilize a viral gene in the presence of activated RAS, MAPK signaling. To prove that we can achieve a similar degree of specificity by the COX-2 3' UTR in human tumor cell lines and this specificity is not transgene specific, we tested the ability of COX-2 3'UTR to regulate cytokine gene expression in human primary and tumor cell lines. The COX-2 3' UTR was inserted downstream of a human cytokine cDNA GM-CSF (GMCSF-COX2 3'UTR). Two human tumor cell lines (LnCap and HCT116) with elevated level of activated MAPK and a human primary cell line (BEAS) with basal level of MAPK (Figure 3B, chapter 4, page-152) activity were then transfected with the pCR3.1-hGMCSF vectors with and without the COX-3 3' UTR, allowed to recover for 24 hours after transfection, and assayed for GM-CSF expression by ELISA. In the presence of the COX-2 3' UTR, significant increase in GMC-SF production over time was detected only in the two tumor cell lines (**Figure 5A**). Although there was some GM-CSF expression detected in the primary cell line, the expression level did not increase over time. At 96 hours post transfection the tumor cell lines were able to produce about two fold more GM-CSF than the primary cells (**Figure 5A**).

3.2.5 Effects of copy number of COX-2 3'UTRs on tumor cell selective gene expression

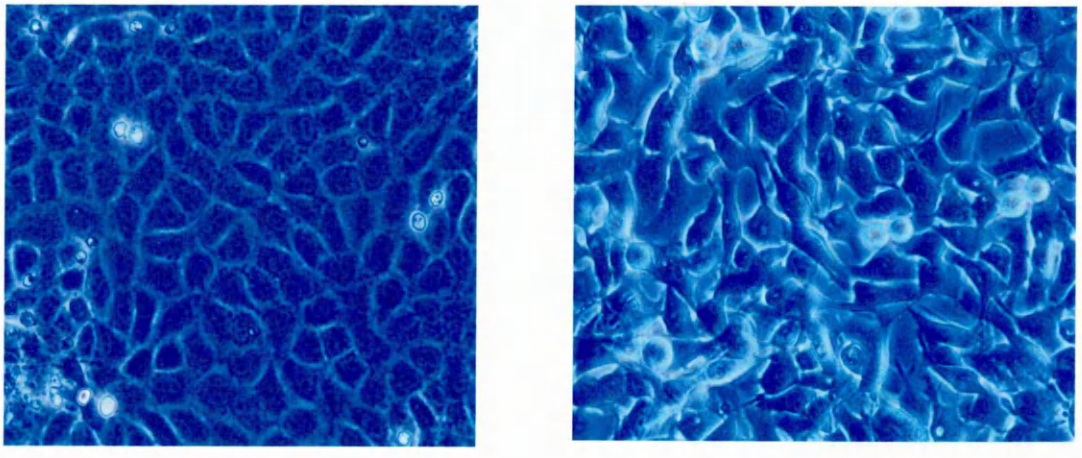
To test weather increasing the length or the number of repeated copies COX-2 3'UTR element will increase the tumor cell selectivity, we generated a series of reporter vectors carrying 1, 2 and 3 copies of COX-2 3'UTR ligated downstream of the GM-CSF transgene. We transfect an equal amount of these plasmids into the human colorectal cancer cell line HCT116 and their effects on the GM-CSF expression by ELISA over time. As shown in figure 5B, the insertion of multiple copies of the COX-2 3'UTR did not improve its ability to express the GM-CSF gene selectively in the tumor cells. There was an inverse correlation between the number of copies of the COX-2 3'UTR and the amount of GMC-SF expression overtime in the tumor cell.

Figure 1

1A.



1B.



(-)IPTG (+)IPTG (48h)

1C.

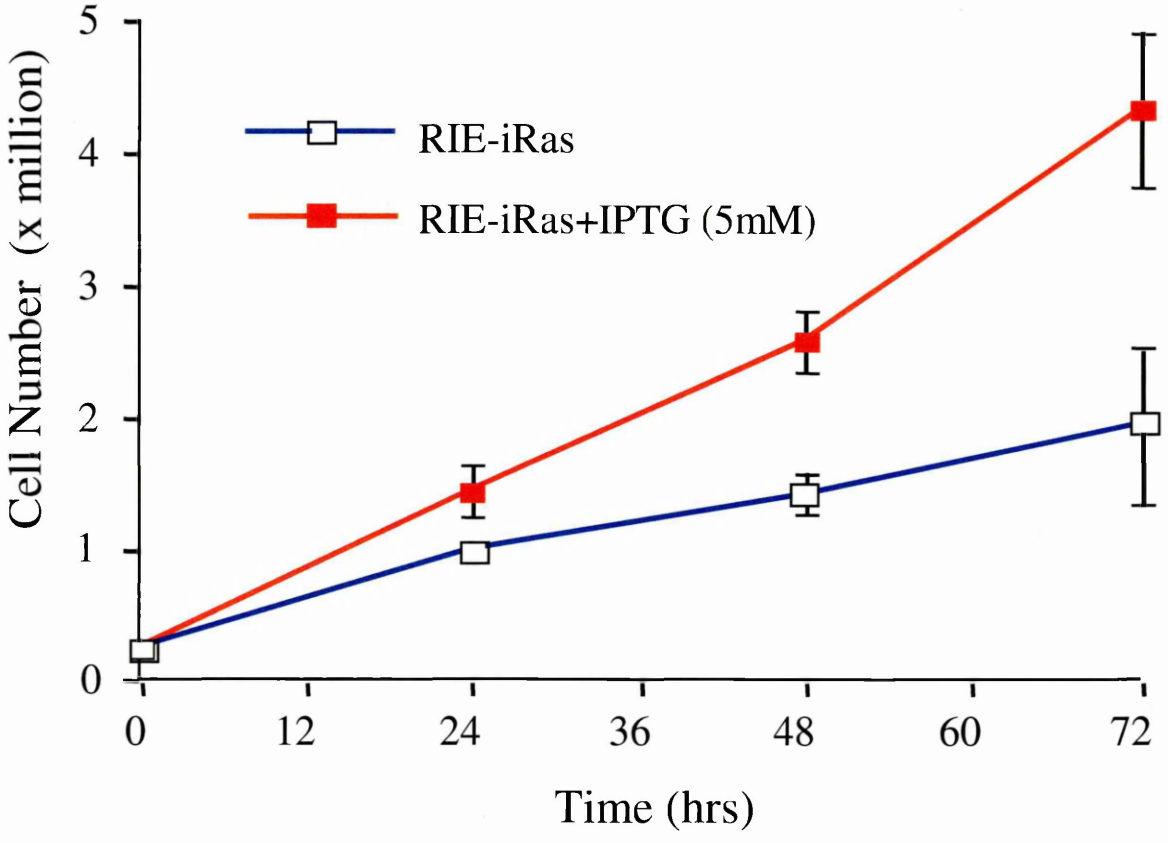


Figure 1. A. Growth of RIE-iRAS cells in 5mM IPTG in culture leads to induction of Ha-Ras^{Val12} as assessed by Western Blot. **B.** Morphological transformation of RIE-iRas cells. The cells were treated with 5mM IPTG 0 and 48 h. the pictures were taken by using an inverted microscope (original magnification X200) **C.** Induction of the Ha-Ras^{Val12} gene in RIE-iRAS cells by 5mM IPTG induces transformation as seen by increased growth rate in culture.

Figure 2

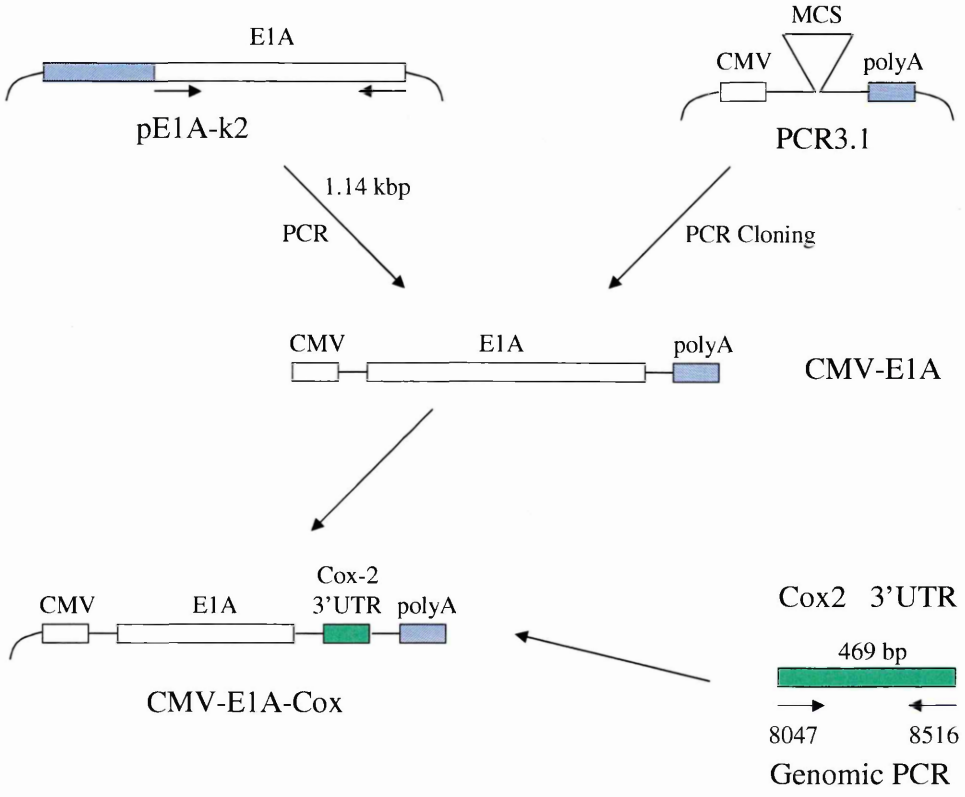
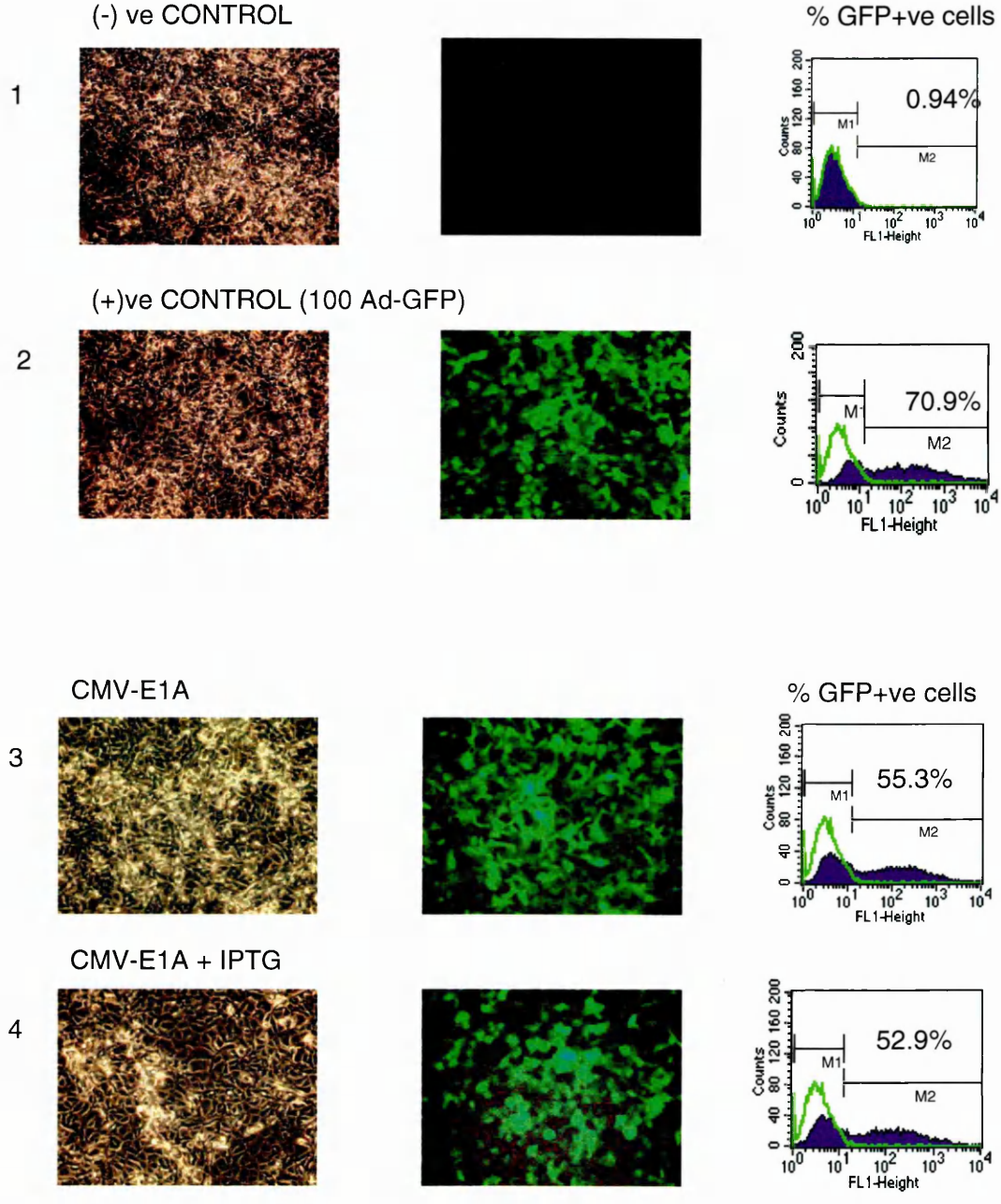


Figure 2. Construction of plasmids CMV-E1A and CMV-E1A-COX. The adenoviral E1A cDNA was PCR cloned into the expression plasmid pCR3.1 to generate CMV-E1A. A 469bp fragment of the 3'UTR of the COX-2 gene was cloned from genomic DNA by PCR and ligated downstream of the E1A gene to give CMV-E1A-COX.

Figure 3

3A.


(-) ve CONTROL
% GFP+ve cells
1
0.94%
M1
M2
Counts
FL1-Height
(+)ve CONTROL (100 Ad-GFP)
2
70.9%
CMV-E1A
% GFP+ve cells
3
55.3%
CMV-E1A + IPTG
4
52.9%

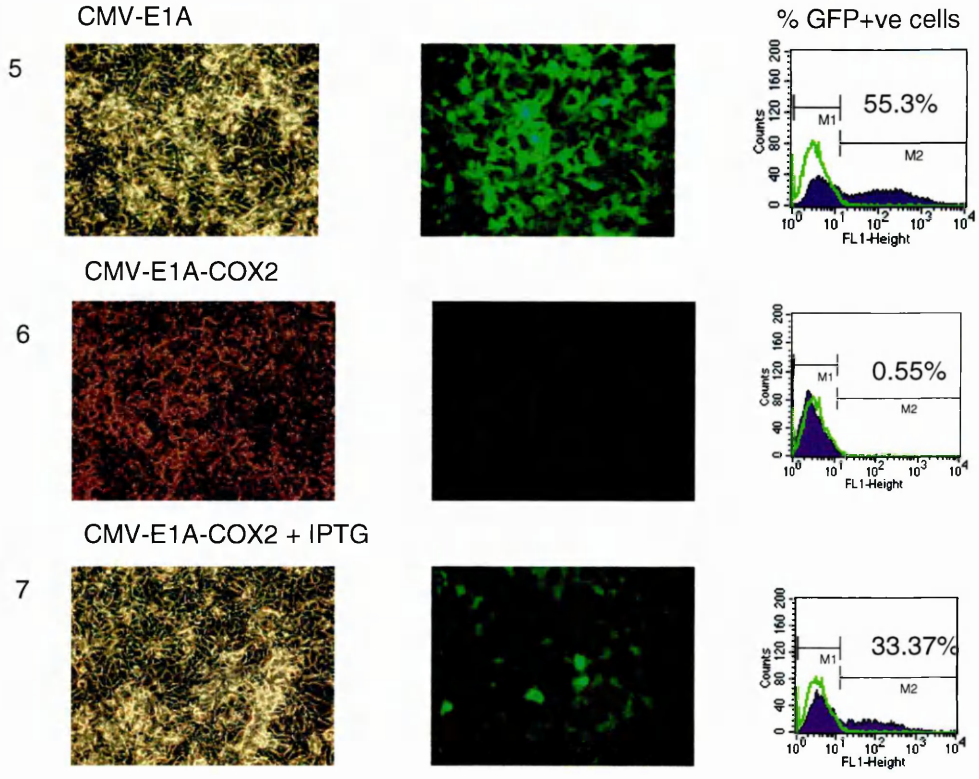


Figure 3. Induction of the Ha-Ras^{Val12} gene in RIE-iRAS cells stabilizes E1A expression sufficiently to allow mobilization of a replication incompetent adenoviral vector. **A.** 5X10^5 RIE-iRas cells were plated in the presence or absence of IPTG(5mM). 24 hours following transfection with 1.0 μg of CMV-E1A (3-5) or CMV-E1A-COX (6-7) DNA the cells were infected with a replication-defective Ad-GFP (10 m.o.i.) vector (3-7). Spread of the GFP reporter gene through the culture was assayed with time using FACS analysis. Expression of GFP is shown at 72 hours following Ad-GFP infection. Control cells were transfected with an irrelevant plasmid and infected with Ad-GFP. Results shown are representative of four different experiments.

3B.

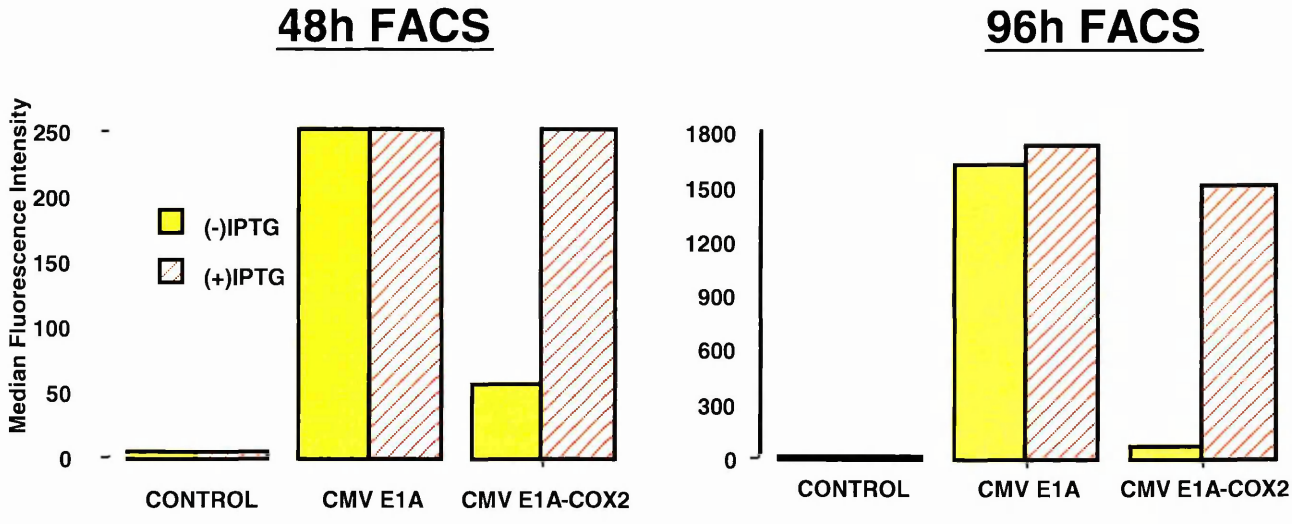


Figure 3B. HT1080 cells were exposed to 72 hour supernatants following Ad-GFP infection of the RIE-iRAS cells transfected with either CMV-E1A or CMV-E1A-COX and treated +/- IPTG as shown. Infected HT1080 cells were analyzed by FACS to detect levels of Ad-GFP.

Figure 4

4A.

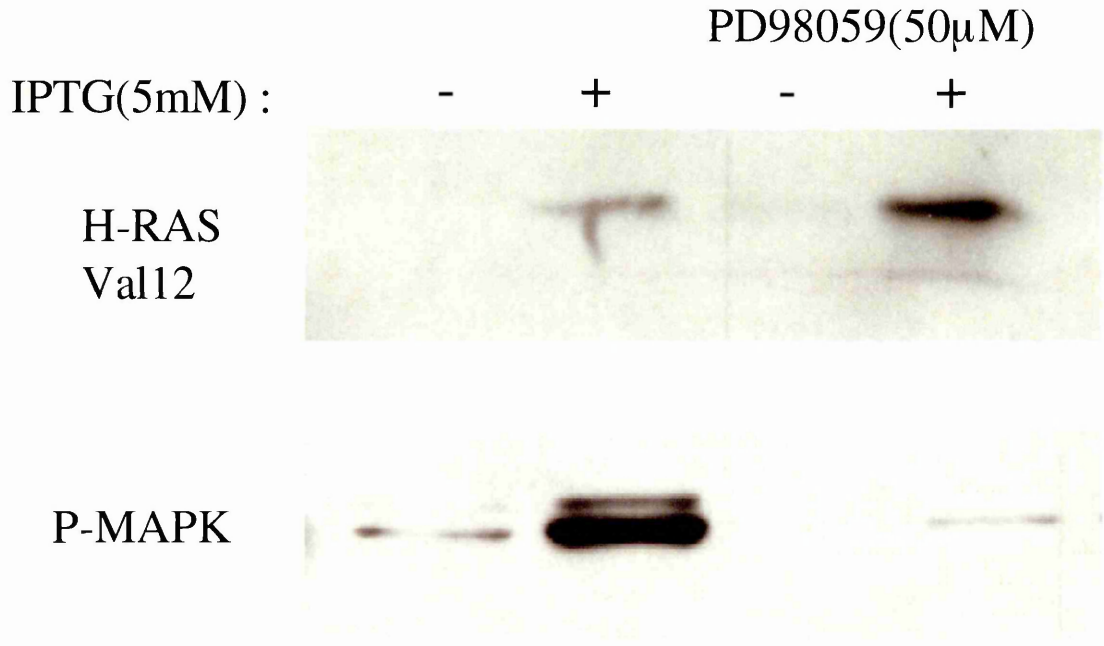


Figure 4. A. Levels of Ha-Ras^{Val12} and activated P-MAPK were determined by western blot in RIE-iRas cells treated with IPTG in the presence of DMSO or PD98059 (50μM) for 48 h.

4B.

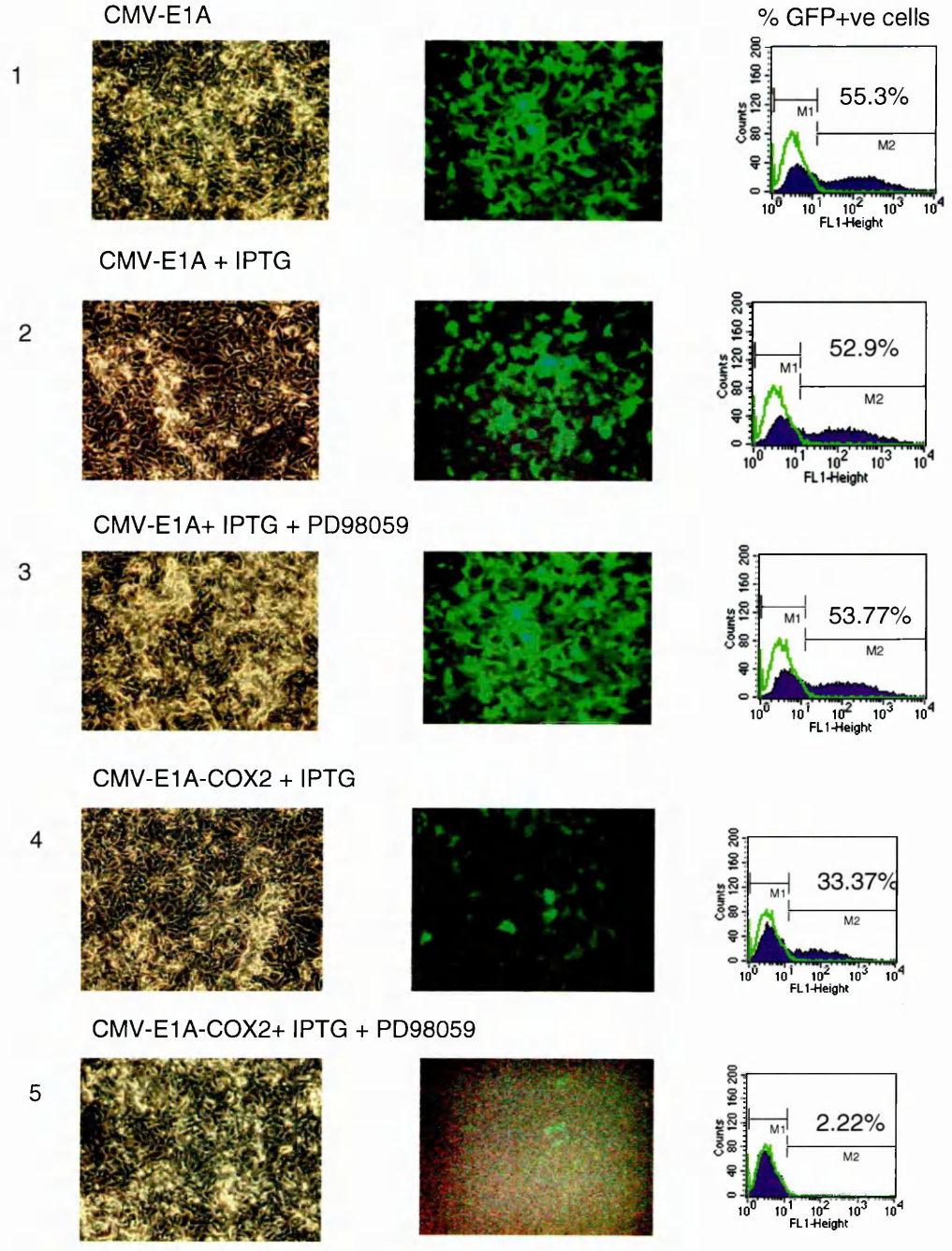

CMV-E1A
% GFP+ve cells
1
55.3%
M1
M2
Counts
FL1-Height
CMV-E1A + IPTG
2
52.9%
CMV-E1A+ IPTG + PD98059
3
53.77%
CMV-E1A-COX2 + IPTG
4
33.37%
CMV-E1A-COX2+ IPTG + PD98059
5
2.22%

Figure 4. Inhibition of Ha-Ras^{Val12}-induced P-MAPK activation by PD98059 blocks COX-2 3'UTR-mediated stabilization of E1A expression. **B.** Mobilization of an Ad-GFP replication-incompetent vector through RIE-iRAS cells treated as shown was measured using fluorescence and FACS analysis. RIE-iRAS cells were transfected with (1-3) CMV-E1A or (4-5) CMV-E1A-COX (1.0 μg), infected with Ad-GFP at an m.o.i. of 10 and then treated with IPTG (2-5) or IPTG and PD98059 [50μM] (3 and 5). 72 hours later, the number of cells expressing GFP (a measure of the mobilization of the Ad-GFP by E1A expression) was measured as shown. For represents uninfected cells and cells infected with $8x10^5$ pfu Ad-GFP, see figure 3A (1 and 2).

Figure 5

5A.

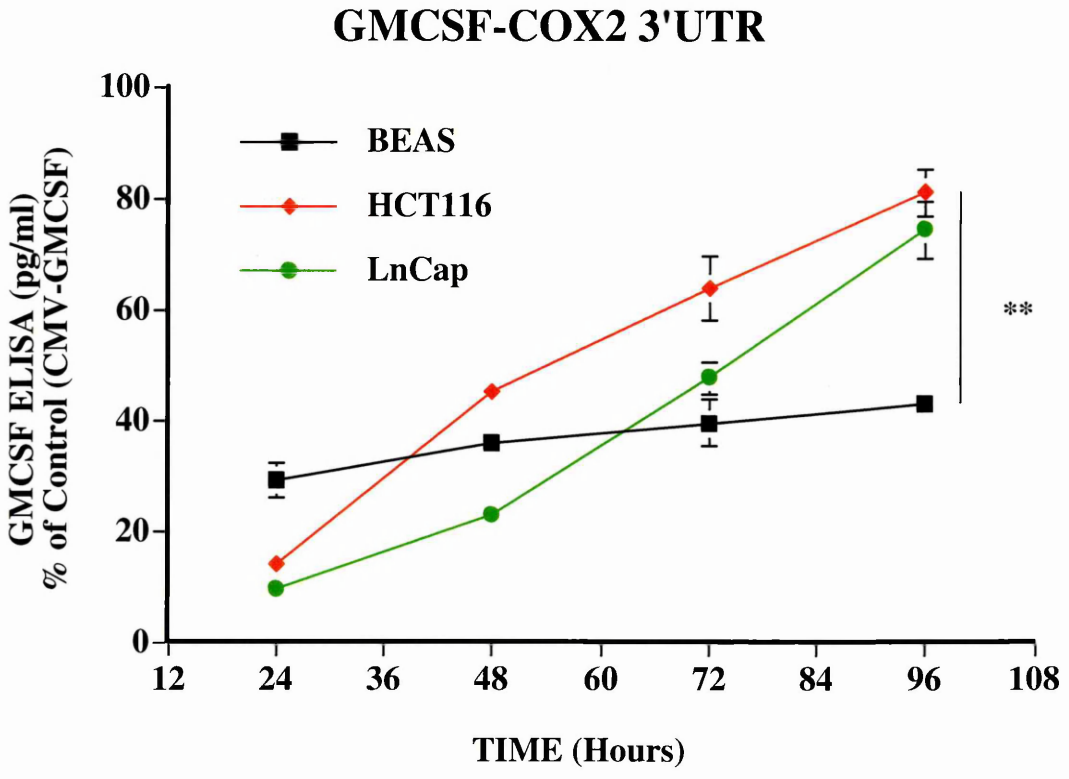


Figure 5. A. COX-2 3' UTR able to regulate GM-CSF expression in different human tumor cell lines with elevated level of activated Ras/MAPK oncogenic signal. One primary cell line BEAS with low level of activated Ras/MAPK and two tumor cell lines (HCT116 and LnCap) with elevated level of activated Ras/MAPK were transiently transfected with pCR3.1 plasmids containing GM-CSF cDNA alone or ligated to COX-2 3'UTR. The supernatant was collected every 24 hours for 96 hours and assayed for the GM-CSF by ELISA at day 5. Results of different treatment groups were expressed as a percentage of the control plasmid (pCR3.1 GM-CSF)(**p<.025).

5B.

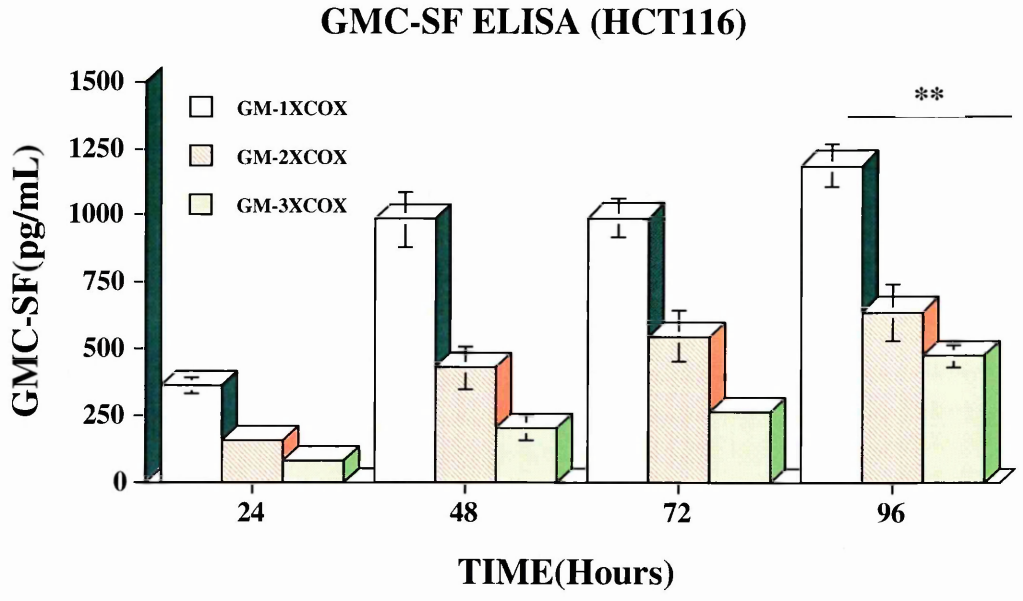


Figure 5. B. Effect of COX-2 3'UTR dosage on the tumor cell selective transgene expression. HCT116 cells transfected with pCR3.1 plasmid carrying GM-CSF transgene, which was ligated to multimers of COX-2 3'UTR. The supernatant from the transfected cells were collected every 24 hours for 96 hours and assayed for the GM-CSF by ELISA at day 5. (**p<.012).

3.4 Discussion

In this chapter, we showed that inserting 469 bp of the ARE-containing 3'untranslated region from a tumor associated gene COX-2 downstream of the adenoviral early gene E1A, is sufficient enough to restrict E1A expression in COX-2 overexpressed transformed cells. This restricted expression of E1A was able to support *in-trans* tumor cell selective replication of a replication defective adenoviral vector. Tumor specific Ras signaling is essential for the COX-2 3'UTR-mediated tumor cell selective expression of the E1A gene and inhibition of MAP kinase, a downstream effectors molecule of Ras, which could abolish the E1A-COX2 dependent restricted adenovirus replication in tumor cells.

The association between aberrant COX-2 expression and carcinogenesis is well documented. A significant amount of both experimental and epidemiological data identified COX-2 as an important player in cancer initiation and progression. Deregulated induction of COX-2 in the tumor microenvironment is initiated by both transcriptional and post-transcriptional mechanisms. The post-transcriptional mechanism of COX-2 induction is mainly mediated by the highly conserved AU-rich *cis*-acting sequence, which is located in the 3'UTR of the COX-2 mRNA. In cancer cells, the presence of constitutively active Ras-MAPK signaling selectively stabilized very unstable COX-2 mRNA and increased COX-2 expression through the ARE of the COX-2 3'UTR. The results presented in this chapter show some *in vitro* evidence for the use of tumor specific COX-2 ARE-activity to stabilize mRNA and thus achieve tumor targeted therapeutic gene expression.

It has been previously reported that the 3' UTR of the COX-2 alone can destabilize reporter luciferase and α-globin mRNAs, but also can stabilize the same mRNA in the presence of activated Ras signaling (Zhang et al., 2000) (Sheng et al., 2000). The 3'UTR of

the COX-2 mRNA is about 1455-nucleotide and containes 12 highly conserved AU-rich elements (AUUUA repeats) (Jones et al., 1993). Six of these AU-rich elements (ARE) are cluster in the 116-nucleotide sequence located close to the COX-2 termination codon and play an important role in regulation of COX-2 expression by altering the mRNA stability and protein translation (Dixon et al., 2000). Based on this information, we cloned 469-nucleotide COX-2 3' UTR downstream of the adenovirus E1A gene, which contains these six AREs. One of our specific aims of this project is to develop a conditionally replicating adenoviral vector by using the COX-2 3'UTR. The adenoviral E1A gene has been extensively and successfully used for this purpose and we choose to use this transgene to test our hypothesis by using *in vitro* mobilization assay, which measures the ability to support the replication and mobilization of a E1A deleted replication-incompetent adenovirus vector expressing marker gene GFP (AdGFP) in the RIE-iRas cells. The E1A protein expressed from the control plasmid CMV-E1A was able to supports considerable mobilization of the incoming Ad-GFP vector independent of the inducible Ha-Ras^{Val12} (**Figure 3A- 4 & 5**). But in the presence of COX-2 3'UTR, the ability of the E1A to support Ad-GFP mobilization was decreased about 100 fold (**Figure 3A-6**) and the mobilization of Ad-GFP was partially regained about 60 fold once the Ha-Ras^{Val12} was induced by IPTG (**Figure 3A-7**). Thus, E1A expression in the presence of COX-2 3'UTR was dependent on the IPTG induced Ha-Ras^{Val12} expression and able to support *in-trans* the replication and mobilization of a replication defective AD-GFP vector.

To test our hypothesis, we selected a model cell system where the normal rat intestinal epithelial cell (RIE-1) can be transformed by inducible expression Ha-Ras^{Val12} (Sheng et al., 1997a). The induction of H-Ras in the RIE-iRas by IPTG led to a COX-2 expression,

which is a direct consequence of Ras-mediated COX-2 mRNA stabilization (Sheng et al., 2000). Although, this model cell system is artificial and does not have all the characteristics of other human cancer cells, it was an ideal *in vitro* system to evaluate our hypothesis because of its ability to induced COX-2 expression via oncogenic Ras-dependent post-transcriptional mechanisms (Sheng et al., 1997a). Our results suggest that the suppression of Ad-GFP mobilization by the COX-2 3'UTR element in the absence of IPTG-induced Ha-Ras^{Val12} is relatively stronger than the activation of Ad-GFP mobilization after the induction of Ha-Ras^{Val12} (100 fold decrease vs. 60 fold increase). Also, we do not know what is the minimal level of Ras activity required for the adequate amount of E1A expression to support Ad-GFP expression. A more sensitive system such as rapamycin inducible system could be used to express Ras more controlled manner and address this issue. This information will be important to identify what kind of cancer cells we will be able to target with the COX-2 3'UTR-mediated tumor selective mRNA stabilization.

PD98059, a highly specific chemical inhibitor for Ras/MAP kinase signaling cascade, can prevent activation of a downstream effectors MAP kinase kinase-1 by blocking access to activating enzymes (Alessi et al., 1995). It has been shown that in colorectal cancer cell lines, constitutive activation of MAP kinase pathway induces elevated levels of COX-2 expression (Dixon et al., 2000) and blocking MAPK activation by PD98059 decrease the level of COX-2. In RIE-iRAS cells, expression of the activated Ras oncogene, even at relatively low levels, induces high levels of MAPK activation and PD98059 can effectively block the MAPK activation (Figure 4A). When we treat IPTG-induced RIE-iRAS cell with PD98059, the ability of the COX-2 3'UTR to mobilize Ad-GFP is significantly reduced even in the presence of the activated Ha-Ras^{Val12} (Figure 4B- 3 & 5). The MAPK inhibitor

does not have any effects on the controlled plasmid CMV-E1A-mediated Ad-GFP vector mobilization and replication (**Figure 4B- 2 & 3**), which rules out the possibility of any nonspecific effects of the PD98059. These experiments demonstrate the requirement of the activated Ras/MAPK signaling cascade for the COX-2 3'UTR-mediated selective E1A expression and Ad-GFP vector mobilization in the IPTG induced Ras-transformed cells.

The next question we asked has to do with selectivity of the COX-2 3' UTR to regulate gene expression in human tumor and primary cell lines. Also, to prove that the COX-2 3'UTR can be used to regulate the selective expression of other therapeutic genes in tumor cells, we inserted the COX-2 3'UTR downstream of the human cytokine gene GM-CSF and tested the effects of the COX-2 3'UTR on GM-CSF expression. Compared to primary cells, the COX-2 3'UTR was more active in the two different human cancer cell lines we tested (**Figure 5A**). GM-CSF expression from CMVp-GMCSF-COX2 plasmid was increased over time and reached about 80% of the control CMVp-GM, in both of the tumor cell lines. In the primary cells BEAS-2B, in the presence of the COX-2 3'UTR, GM-CSF expression never reached over 40% of the controlled plasmid. From these results we can conclude that the COX2 3'UTR is more active in human tumor cells than in normal cell and can regulate another transgene in a tumor cell selective manner. Next, we tried to improve the ability of COX-2 3'UTR element to stabilize specific mRNA selectively in the tumor cells. It has been previously reported that a targeting element such as COX-2 3'UTR can be used in multiple copies in a row in order to improve their ability to regulate gene expression in specific environments. For examples, a hypoxia-responsive element (HRE) derived from the 5'UTR of the human vascular endothelial growth factor improved its induction ability as the number of copies of HRE increased at tumor hypoxia (Shibata et

al., 2000). Based on these reports, we thought we might be able to improve the Ras responsiveness of the COX-2 3'UTR by increasing the number of copies of the ARE-element from one to three. We constructs and compared the Ras responsiveness of a series of constructs carrying 1, 2 and 3 copies of 469 bp COX-2 3'UTR downstream of GM-CSF gene expressed under CMV promoter and test these constructs in the HCT116 by transient transfection and monitored the GM-CSF expression by ELISA. By increasing the number of 3'UTR element, we saw the GM-CSF expression was significantly suppressed in the Ras activated HCT116 cancer cells. Compared to one copy of COX-2 3'UTR, there was about 50% reduction of GM-CSF expression with three copies of COX-2 3'UTR at 96 hours post transfection (**Figure 5B**). So the increased copy number of COX-2 3'UTR has a negative effect on tumor selective transgene expression.

Data from Chapter 3 indicates that tumor selective mRNA stabilization can be used as a novel strategy in tumor-selective gene expression for cancer gene therapy. We believe that such construct can be used to drive therapeutic genes or replicative essential viral gene to generate conditionally replicating viral vectors. We propose to exploit the tumor selective mRNA stabilization via COX-2 3' UTR by fusing it with the adenovirus early essential gene E1A, thereby obtaining a conditionally replicating adenovirus vector, which will preferentially replicate in the RAS transformed cells. To that end, the priority is to develop a conditionally replicating adenoviral vector by using the COX-2 3'UTR and this will be the focus of the following chapter.

The data presented in this chapter from parts of the following paper:

Ahmed A, Thompson J, Emiliusen L, Murphy S, Beauchamp DR, Suzuki K, Alemany R, Harrington K and Richard G. Vile. A Conditionally Replicating Adenovirus Targeted to Tumor Cells Through Activated RAS/P- MAPK-Selective mRNA Stabilization. *Nature Biotech*; 2003 Jul; 21(7): 771-7.

Chapter 4

A Conditionally Replicating Adenovirus Targeted to Tumor Cells Through Tumor Cell Selective mRNA Stabilization

4.1 Introduction

As described in previous chapters, COX-2 3'UTR can be used *in vitro* to regulate gene expression by tumor cell selective mRNA stabilization mechanisms and to control replication of an adenoviral vector. In order to assess the utility of the COX-2 3'UTR as an element to control viral replication when it is inserted in to the viral genome, it was necessary to develop adenoviral vector with E1A-COX2 3'UTR. In this chapter, we focus on the development and the characterizations of adenoviral vector carrying the E1A gene ligated to 469 bp COX-2 3'UTR element.

Gene therapy for cancer has emerged as a targeted approach that would significantly reduce undesired side effects. In this approach, it is essential to ensure that the vectors used for gene therapy be targeted very efficiently in order to reduce toxicity compared to conventional therapies (Verma & Somia, 1997). In general, tumor-selective gene expression from adenoviral vectors (replication competent or incompetent) has been achieved through transcriptional regulation using selective promoters driving either essential replicative (Sadeghi & Hitt, 2005) or therapeutic genes (Bilsland et al., 2003). The promoter/enhancer used for these vectors is derived from genes whose expression is selectively upregulated in tumor cells as opposed to normal counterparts (Matsubara et al., 2001) (Wirth et al., 2003) However, it is also clear that tissue specific promoter/enhancer elements inserted into adenoviral genomes are affected by viral enhancers requiring the addition of other insulator elements, thereby complicating the efficacy of such approaches (Sadeghi & Hitt, 2005) (Wirth et al., 2003). In contrast, the control of mRNA stability of tumor-associated proteins has not yet been exploited for the design of tumor specific replicating viruses. In the previous chapter, we demonstrated that in vitro the adenoviral

E1A gene can be selectively expressed by the COX-2 3'UTR element in the tumor cells with an elevated level of Ras/MAPK signaling and this tumor specific E1A expression can support replication and mobilization of a replication incompetent adenoviral vector. Based on this, we hypothesize that we might be able to use tumor cell selective stabilization of mRNA as a novel mean to control viral gene expression and to develop a conditionally replicating viral vector for cancer gene therapy. We describe here the construction of a conditionally replication competent adenoviral vector in which expression of the essential E1A gene is regulated by ligation to the 3'UTR of the COX-2 gene, allowing RAS/P-MAPK-specific stabilization of the mRNA. This is the first description of a replicating (adeno)virus whose tumor selectivity is based upon control of gene expression at the level of mRNA stability. This strategy has great potential for expansion since there are many different genes whose 3'UTRs control selective mRNA stability under different physiological, pathological and tumor-associated conditions

4.2 Results

4.2.1 Construction of recombinant adenoviral shuttle vector expressing E1A ligated to COX-2 3'UTR

The 469bp human COX-2 3'UTR was cloned as described in the previous chapter. The Ad-E1A-COX is an *E1/E3* deleted, serotype 5 vector that contains the cytomegalovirus (CMV) immediate-early gene promoter-enhancer driving the adenovirus E1A cDNA (1100bp) which is fused with COX-2 3' UTR (469bp). This vector was constructed by using an AdEasy kit, according to the manufacturer's protocol (Qbiogene,CA). Briefly, E1A-COX-2 gene was PCR cloned from plasmid pE1A-K2-COX and inserted into the transfer plasmid, pShuttle (AdEasy kit, Qbiogene,CA) by using the unique *Hind*III-*Eco*RV sites. The resulting plasmid (pShuttle-E1A-COX) was then linearized with *Pme*1 and co-transfected into *E.coli* strain BJ5183 together with pAdEasy-1 (Qbiogene,CA), the viral DNA plasmid. The recombinant adenoviral construct was then cleaved with *Pac*I to expose its Inverted Terminal Repeats and transfected into 293A cells to produce viral particles. The selective vector clone was then plaque purified at least three times before it was used in experiments. For *in vivo* experiments, the virus was purified on cesium chloride gradient columns.

4.2.2 Confirmation of recombinant adenovirus by Hirt extraction

Incorporation of the E1A gene with or without COX-2 3'UTR within the recombinant adenovirus was confirmed by Hirt extraction. The recovered DNA was analyzed in a PCR: 2 μl of sample DNA was added per PCR reaction mix. Specific primers that were design to anneal in the shuttle vector just outside the inserted transgene were used to test for incorporation of the E1A gene. PCR were performed by using AmpliTaq with the following condition: 94° for 10 minutes, then 30 cycles of 94° for 1 minute, 55° for 1.5

minutes, 72° for 2 minutes. Samples from the PCR were run on the gel (See Figure 1B). The PCR detects the incorporation of 1.0 kb fragment corresponding to E1A gene and 1.5 kb fragment corresponding to E1A-COX2 3'UTR in their designated adenovirus.

4.2.3 E1A expression can be destabilized within an adenoviral genome by the COX-2 3'UTR and re-stabilized in the presence of activated RAS and high levels of P-MAPK

Next, we incorporated these E1A and E1A-COX cassettes into E1A-deleted adenoviral genomes using a protocol to generate recombinant adenoviral vector as described in the method and materials section. Both the Ad-E1A and Ad-E1A-COX viruses recovered from transfection of 293 cells were purified (**Figure 1B**) and used to infect RIE-iRAS cells in the presence or absence of IPTG with 10 MOI (described in the previous chapter). 7 days post-infection, the surviving cells from infected culture were counted. As shown in figure 2A, replication and oncolysis of Ad-E1A-COX in RIE-iRAS cells was heavily dependent upon IPTG induction of Ha-Ras^{Val-12}. In addition to the cytotoxicity assay described in **Figure 2A**, we also assayed replication of the Ad-E1A-COX virus directly in infected RIE-iRAS cells (+/-) IPTG by measuring the viral titer (**Figure 2B**). Ad-E1A-cox-infected RIE-iRAS cells in the presence of IPTG consistently produced in excess of 3 logs more virus (10^5 plaques per 10^5 lysed infected cells) per cell than the same cultures in the absence of IPTG induction (10^2 plaques per 10^5 lysed infected cells) (**Figure 2B**). We also wanted to confirm that the effects we observed in the model RIE-iRAS system were reproducible in human cell lines with different levels of RAS or P-MAPK activity. Therefore, the levels of P-MAPK in several different human cell lines were measured by Western blot analysis (**Figure 3B**). Of these lines, uninduced RIE-iRAS, U118 glioma and the normal epithelial BEAS cell lines expressed low or undetectable levels of P-MAPK. The remainder

expressed moderate (HT1080, U87, U251 and HCT116) or high (IPTG-induced RIE-iRAS, LnCap and PC3) levels of P-MAPK (**Figure 3B**). These lines were infected with the Ad-E1A or Ad-E1A-COX viruses (at a lower m.o.i. than with the RIE-iRAS line because of the improved ability of human lines to support adenoviral replication). 7 days following infection with an m.o.i. of 0.1, surviving cells were counted (**Figure 3A**). The wild type E1A gene supported ongoing viral replication that caused lysis and killing of every cell line although the efficacy of the wild type virus was reduced in the human prostatic line PC3 (**Figure 3A**). In contrast, the replication of Ad-E1A-COX was much more heavily dependent upon the cell line; in general, oncolysis correlated very closely with the line's P-MAPK activity status. Thus, cultures of normal bronchial epithelial cells (BEAS) with no detectable activated P-MAPK were completely eradicated by Ad-E1A virus infection (**Figure 3A**) but Ad-E1A-COX was significantly less toxic to these cells (BEAS cells are very sensitive to adenoviral infection). Ad-E1A-COX also replicated only very poorly relative to the wild type Ad-E1A in the U118 (glioma) (**Figure 3A**) and uninduced RIE-iRAS cells lines (**Figure 2 A&B**). We did observe some killing of U118 cells by Ad-E1A-COX due to the fact that these cells express low, but still detectable, levels of P-MAPK (see, for example, **Figure 5A & B** below). Infection of the U87 glioma line (moderate P-MAPK activity) with Ad-E1A-COX was effective at killing these cells although not as well as the wild type virus. In contrast, one other glioma cell lines U251, the fibrosarcoma HT1080 and colorectal HCT-116 cell lines and two prostate cell lines LnCap and PC3 were as good substrates for replication of Ad-E1A-COX as for Ad-E1A and all 5 lines express elevated levels of P-MAPK. As for the RIE-iRAS cells in **Figure 2A** above, viral burst assays from the infected human cell lines confirmed the cytotoxicity data in that P-MAPK

expressing cells produced in general 3-4 or 2 logs more virus per infected cell in a replication assay (Sheng et al., 2000) than the BEAS or U118 cell lines respectively. In addition, Northern blot analysis confirmed a direct correlation between the levels of oncolysis of different cell lines, the levels of P-MAPK activity and the expression of steady state levels of E1A mRNA species at early time points following infection with Ad-E1A or Ad-E1A-COX (**Figure 4A**).

4.2.4 Ad-E1A-COX is oncolytic *in vivo* against human tumors expressing high levels of P-MAPK activity

Although the RIE-iRAS cell line grew in nude mice, we observed induction of Ha-Ras^{Val-12} within all of the tumors *in vivo* irrespective of whether IPTG was administered. Therefore, to test the selectivity of the Ad-E1A-COX virus, we used tumor lines that are closely matched histologically, but which differ in levels of P-MAPK activity. The glioma cell lines U118 with low level of P-MAPK and U87 or U251 with high level of P-MAPK moderate/high) (**Figure 5A**) were used to test the *in vivo* efficacy and selectivity of the Ad-E1A-COX virus. Western Blotting confirmed *in vitro* that the level of E1A expression supported by Ad-E1A and Ad-E1A-COX infection (**Figure 5B**) reflects very closely the P-MAPK activity of these lines (U118<< U87<U251) (**Figure 5A**) data, which was confirmed at the RNA level by Northern Blotting (data not shown).

There was a significant difference in the growth rate of established U118 tumors following a single intratumoral injection (10^8 pfu) of wild type Ad-E1A compared to tumors injected with PBS ($p<0.001$) (**Figure 5 C & D**). However, injection with Ad-E1A-COX virus gave no statistically significant difference compared to the PBS injected control (**Figure 5 C & D**) in U118 (low P-MAPK activity) tumors. In the U251 model, the oncolytic effects of a

single intratumoral injection of wild type adenovirus were reduced compared to those in the U118 model (**Figure 5 D&E**), but, consistent with the high levels of P-MAPK activity in this tumor (**Figure 5A**), the Ad-E1A-COX virus either matched, the example shown in **Figure 5 C** with U87 model, or exceeded the efficacy of the wild type virus (**Figure 5E** with U251 model). These findings were also confirmed in the second P-MAPK high glioma model, U87. Thus, combining the results of several experiments, the injection of wild type Ad-E1A virus was effective at reducing the size of U118 tumors (>75% reduction in final tumor size relative to PBS injected control tumors). This therapeutic effect was less pronounced in the U87 model (~30% reduction with respect to PBS injected tumors) (**Figure 5C**). Ad-E1A-COX was, however, as effective as wild type adenovirus when used to treat U87 tumors (moderate/high P-MAPK activity) but had no significant effect on the treatment of subcutaneous U118 tumors (**Figure 5C**) (low levels of P-MAPK activity and de-stabilized E1A expression as shown in **Figure 5B**). The fact that Ad-E1A-COX was even more effective than wild type virus in the U251 model (**Figure 5E**), but that Ad-E1A-COX was only similar to wild type virus in efficacy in the U87 model (**Figure 5C**) is consistent with the observation that U251 tumors express somewhat higher levels of P-MAPK than U87 tumors (**Figures 3B** and **5A**) and accordingly support higher levels of adenoviral replication. Taken together, these *in vivo* results are consistent with the *in vitro* data demonstrating a strong correlation between the P-MAPK status of a tumor and its ability to support the replication of the Ad-E1A-COX virus.

4.2.5 AdE1A-COX shows reduced E1A expression in normal tissues following systemic administration in nude mice.

Given the particular sensitivity of the liver as a potential site of toxicity following therapy with adenoviral vectors, we tested whether the selectivity of Ad-E1A-COX for non-transformed cells was also maintained in normal liver *in vivo*. Athymic nude mice were injected intravenously with either wild type Ad-E1A or Ad-E1A-COX virus (10^6 pfu per mouse) in order to infect normal hepatocytes and other tissues. Three days later, livers were removed from the animals and assayed for expression of E1A mRNA by rtPCR. Hepatic expression of E1A could be detected following infection with Ad-E1A virus in both treated mice (**Figure 6A**). However, the presence of the COX-2 3'UTR was sufficient to lower levels of expression of E1A mRNA to below detectable levels in both mice injected with Ad-E1A-COX virus (**Figure 6A**). Serum was also collected from the treated mice and tested for the presence of replicating virus. Serial dilutions of samples plated onto 293 cells indicated that mice treated with Ad-E1A had very low, but detectable titers of circulating virus (**Figure 6B**), presumably as a result of low level replication in the liver or elsewhere. In contrast, no detectable virus could be recovered from either mouse treated i.v. with Ad-E1A-COX virus. Therefore, the presence of the COX-2 3'UTR reduces significantly levels of E1A expression and viral replication in normal – albeit murine - liver tissue.

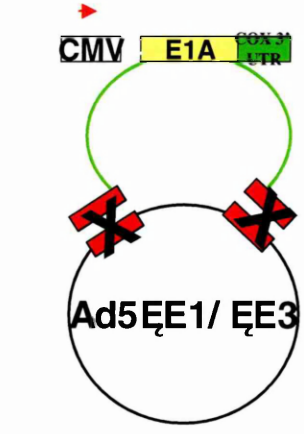
4.2.6 Systemic administration of AdE1A-COX in the immunocompetent murine model induced hepatotoxicity

One of the major problems of using any conditionally replicating adenovirus to treat patients with metastatic disease is the toxicity caused by the viral vector after systemic

administration in the immunocompetent host. So the safety of every viral vector must be evaluated very carefully in the animal model before it is considered for use in humans. To establish the maximum tolerated dose of the AdE1A-COX virus in a immunocompetent murine model, C57BL/6J mice were injected with escalating doses ($1X10^9$-$5X10^9$ pfu/mice) of wild type Ad-5 or recombinant AdE1A-COX virus into the tail vain. The highest dose of Ad-E1A-COX virus evaluated that resulted in 100% survival was $5X10^8$ pfu. Mice receiving $5X10^9$ pfu of Ad-E1A- COX virus as five daily injections of 10^9 pfu resulted in 70% lethality (**Figure 7A**). There was no difference in the overall survival and the lethal dose 50 (LD50) between the groups treated with retargeted Ad-E1A-COX or Wt Ad-5 virus. This was true for all the different doses tested. At the highest dose of $5X10^9$ pfu/mice, the percentage of overall survival from each treatment group was the same (**Figure 7A**), but mice from the group treated with AdE1A-COX virus started to die 5 days earlier than the group with wild type Ad-5. Histophatological analysis of livers from mice that died acutely revealed that the AdE1A-COX virus induced significantly more hepatotoxicity as shown by increased amount of immune infiltrate and severe, diffuse necrosis than the wild type Ad-5 or PBS treated control (**Figure 7B**). Mice that survived the treatment were sacrificed 2 weeks after virus injection and showed normal liver histology. The elevated level of serum liver enzyme ALT was also observed 3 days post i.v administration also confirmed the hepatotoxicity induced by AdE1A-COX virus. But at a later time the ALT level was very similar or even lower (day 14) than the mice treated with wild type Ad-5 (**Figure 7C**). However, there was no statically significant difference in the amount of virus recovered from the liver tissue of the mice treated with either of the virus (**Figure 7D**). If anything, we recovered higher amounts of wt Ad-5 compared to

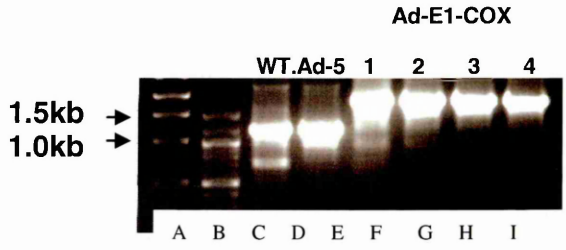
recombinant AdE1A-COX virus at 48 and 72 hours post administration. But because of the smaller sample size (n=3) and large standard deviation, the difference between these two groups was not statistically significant. When we looked at the viral DNA synthesis in the liver tissues of the same mice from these two groups, the AdE1A-COX virus able to synthesize significantly more DNA than the wt Ad-5 virus at 24h post administration. But, in the later time point at 48 and 72h after the i.v. injection, the viral DNA load was much higher in mice treated with wt Ad-5 than the AdE1A-COX virus. There was no difference in the recovered viral titer between the two virus groups in other major organs such as the heart, lung and kidney.

1A



Ad EASY Vector system
Qbiogene

1B



rtPCR for E1A or E1A-COX
from HIRT extracted DNA

Figure 1. A. Schematic diagram of *in vivo* homologous recombination step between a linearized transfer vector carrying adenoviral E1A gene ligated to 469 bp COX-2 3'UTR and an intact supercoiled Ad plasmid in bacteria. **B.** Diagnostic PCR performed on Hirt extracted DNA from HT1080 cells infected with recombinant adenovirus. Lane A: 1Kb ladder, lane B: 100bp ladder, lane C: 1 μg of wild type adenovirus DNA. lane D: Hirt DNA extracted from HT1080 cells infected with 10 MOI of wild type adenovirus, lane E-H: Hirt DNA extracted from HT1080 cells infected with 10 MOI of recombinant Ad-E1A-COX virus.

2A

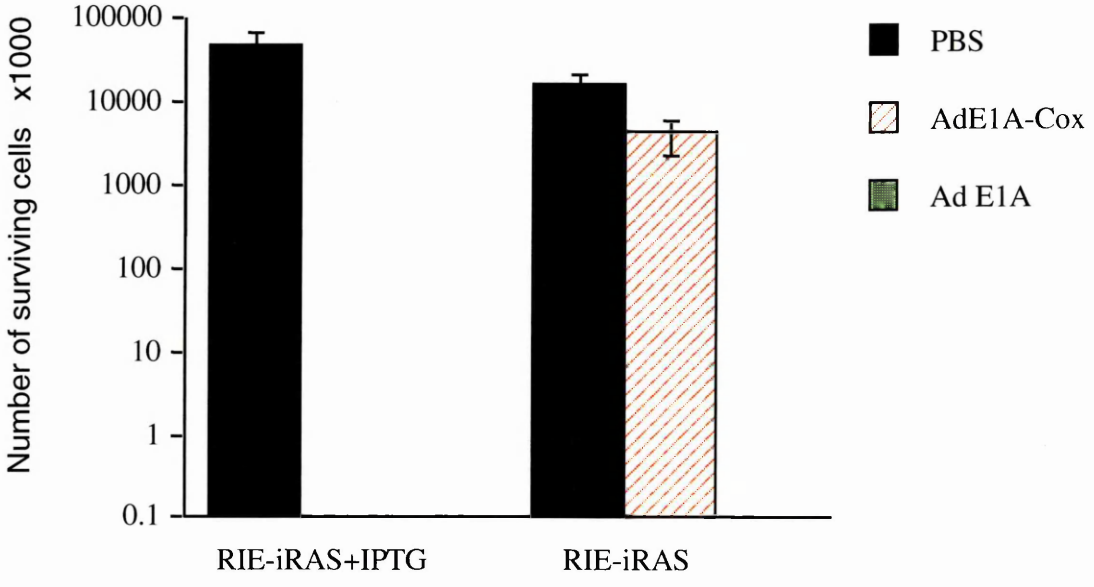


Figure 2. Replication of Ad-E1A-COX correlates with the P-MAPK status of tumor cell lines. **A.** RIE-iRAS cells grown in the presence or absence of IPTG to induce expression of the Ha-Ras^{Val12} oncogene were infected with Ad-E1A or Ad-E1A-COX viruses at an m.o.i. of 10. 7 days later, surviving cells were counted.

2B

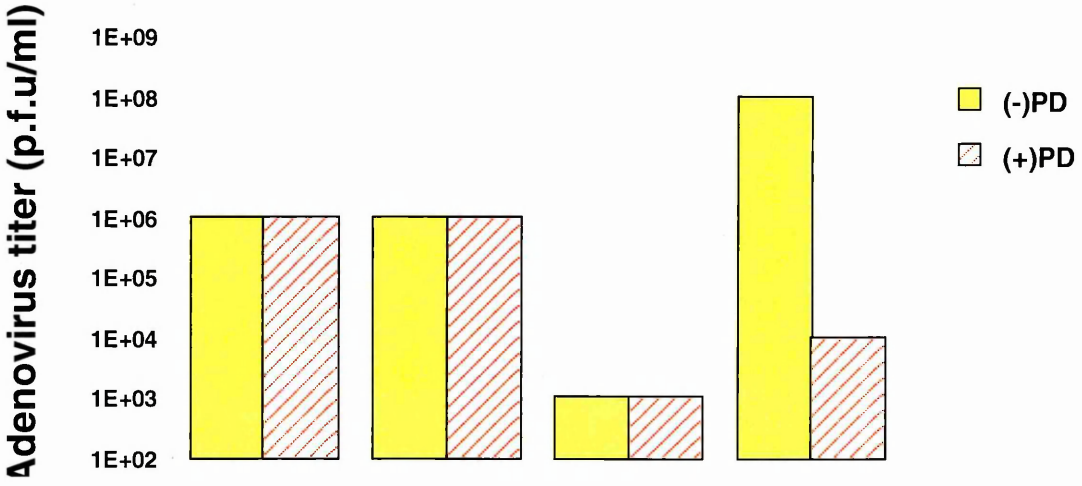
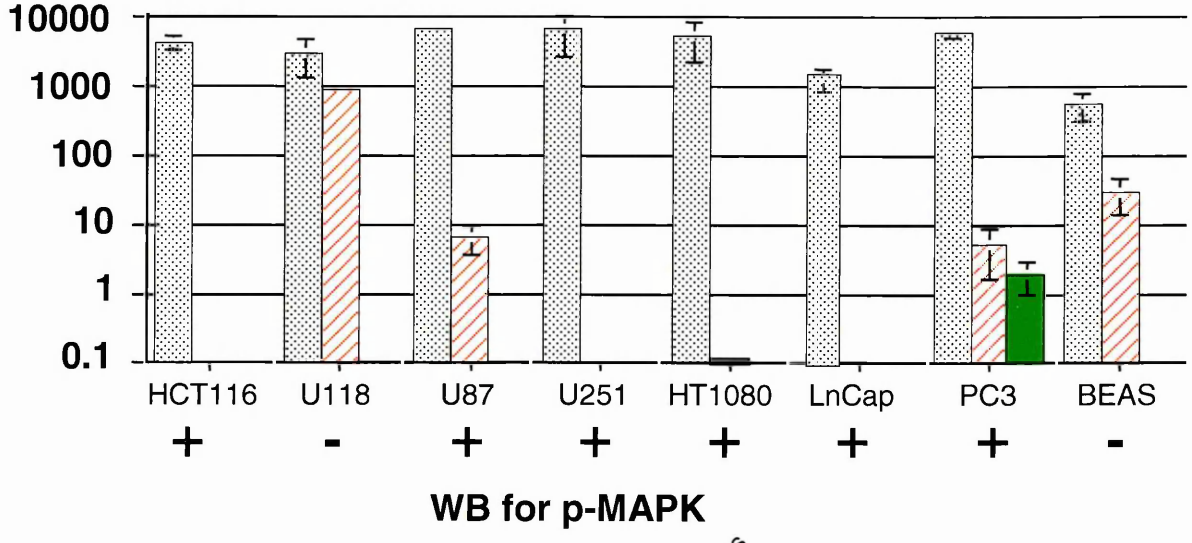


Figure 2B. HT1080 cells were exposed to 72 hour supernatants following Ad-E1A-COX infection of the RIE-iRAS cells transfected with either CMV-E1A or CMV-E1A-COX and treated IPTG or IPTG and PD98059 as shown. Virus titer was measured by plaque assay by using 293A cells.

3A



WB for p-MAPK

3B

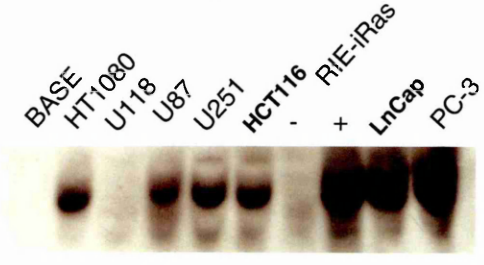


Figure 3A. The range of tumor cell lines, characterized in **B** below were infected with Ad-E1A or Ad-E1A-COX viruses at an m.o.i. of 0.1. 7 days later, surviving cells were counted. **B.** Levels of P-MAPK activity in the cell lines used to assess the *in vitro* cytotoxicity of the Ad-E1A and Ad-E1A-COX viruses were measured by Western blot analysis. Lanes 1-6, human lines: normal bronchial epithelial cells (BEAS); 2, fibrosarcoma, HT1080; 3, glioma U118; 4, glioma U87; 5 glioma U251; 6, colorectal HCT116. Lanes 7 and 8 rat intestinal epithelial RIE-iRAS cells grown in the absence (7) or presence (8) of IPTG to induce expression of Ha-Ras^{Val12}. Lane 9, 10 human prostatic LnCap and PC3 cell respectively.

4A

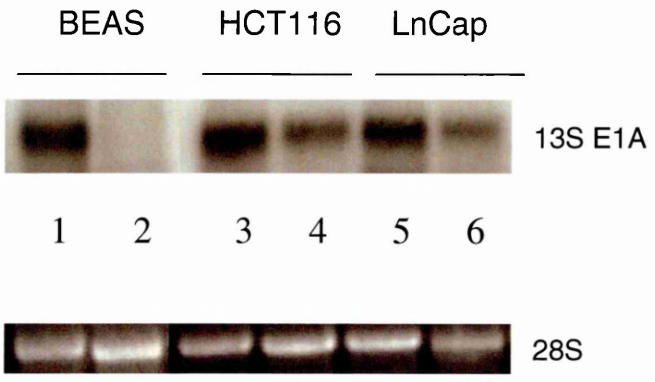


Figure 4. Northern blot analysis for expression of E1A mRNA of representative low P-MAPK (BEAS) or high P-MAPK (HCT116 and LnCap) cell lines infected with Ad-E1A (lanes 1, 3, 5) or Ad-E1A-COX (lanes 2, 4, 6) viruses as described in **Fig. 3.**

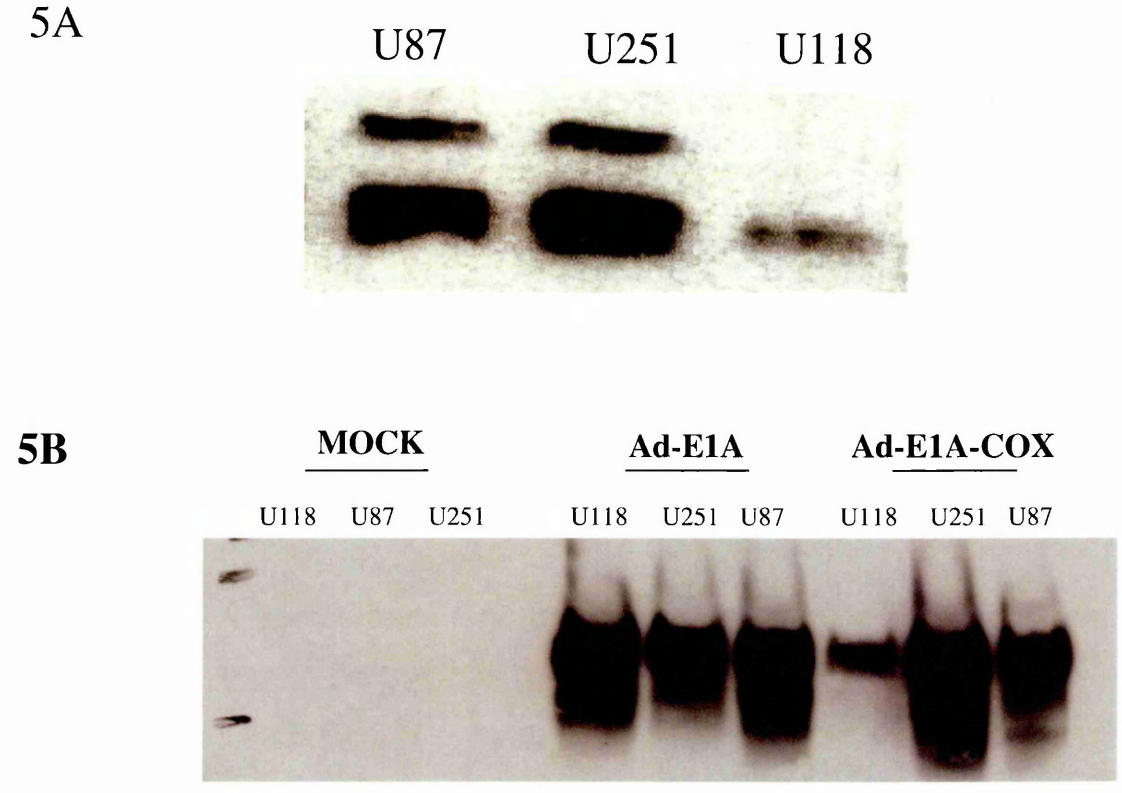
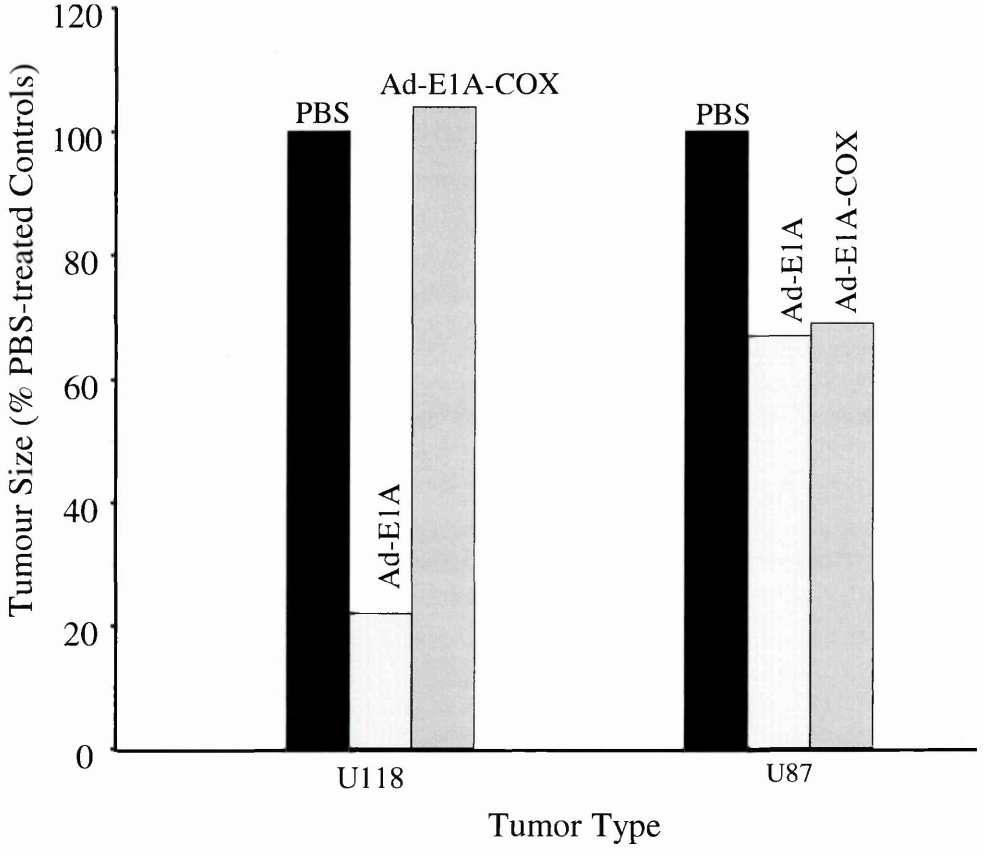


Figure 5. Ad-E1A-COX is selectively oncolytic to tumors expressing high levels of P-MAPK. **A.** Levels of P-MAPK activity in the glioma cell lines U118, U87 and U251 were measured by Western Blotting. **B.** Levels of E1A expression in the glioma cell lines U87, U118 and U251 were assayed by Western Blot 15 hours following infection with Ad-E1A or Ad-E1A-COX viruses at an m.o.i. of 10.

5C


120
100
80
60
40
20
0
Tumour Size (% PBS-treated Controls)
PBS
Ad-E1A
Ad-E1A-COX
PBS
Ad-E1A
Ad-E1A-COX
U118
U87
Tumor Type

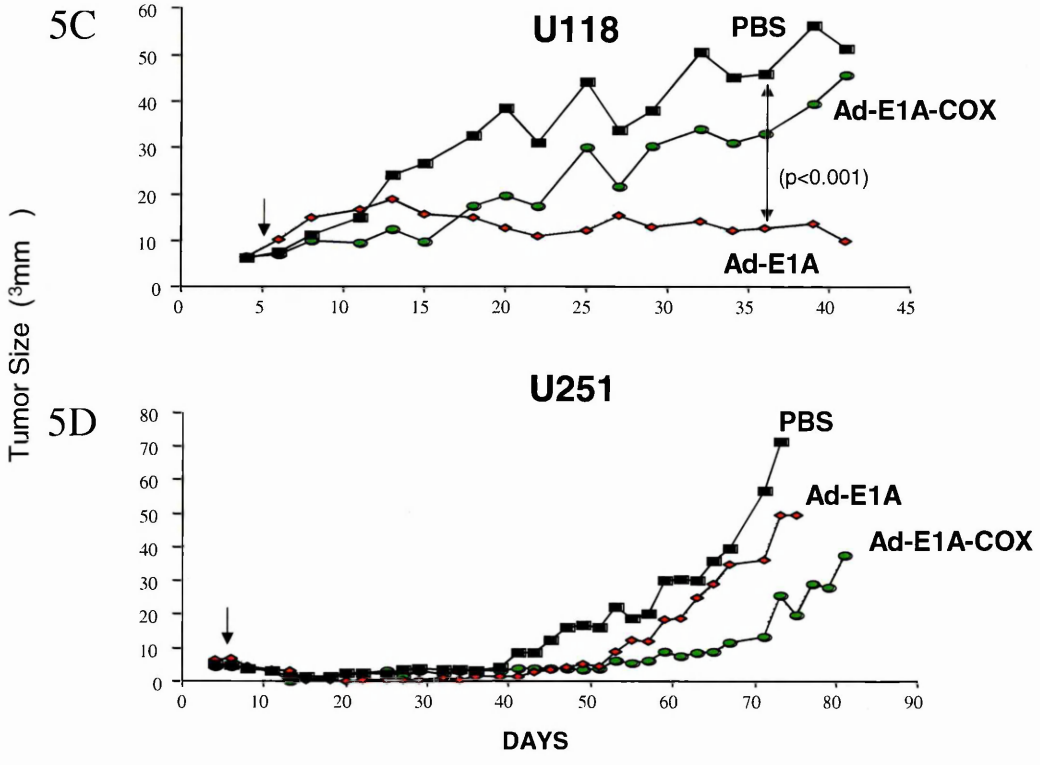


Figure 5C, 5D. U118 (**C**) or U251 (**D**) tumors were implanted subcutaneously in nude mice (10 mice per group) and allowed to develop to size between 0.2-.04 cm. These established tumors were injected directly with equal doses of Ad-E1A, Ad-E1A-COX (10^8pfu) or PBS, in a total volume of100µl and tumor growth was followed with time. **E.** The same experiments as described in **C** and **D** were carried out with the U118 and U87 tumor lines. Experiments were terminated 60 days following virus injection when mean tumor sizes in all groups were measured. Results of different treatment groups, over different experiments, were expressed as a percentage of the mean size of the PBS injected control groups.

6A

Circulating Titre of Virus (pfu/ml)

	i.v. Injection (10^6 p.f.u.)	Blood (day 3)	Liver (day 3)	Liver (day 6)
Ad E1A	Mouse #1	10	-	-
	Mouse #2	10^3	$2 X10^2$	-
Ad-E1A-cox	Mouse #1	0	-	-
	Mouse #2	0	-	-

i.v. administration of $1.0 X 10^6$ pfu of different viruses.

6B

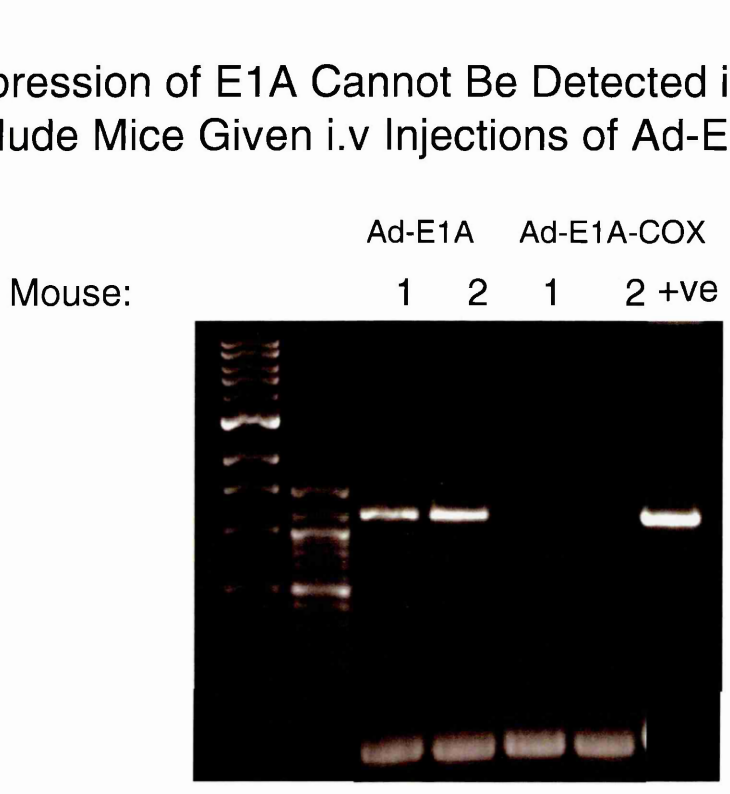


Figure 6. Replication of Ad-E1A-COX cannot be detected following systemic administration. **A.** Mice (2 per group) were injected i.v with Ad-E1A or Ad-E1A-COX virus(10^6 pfu/mouse). Serum from these mice was recovered and plated in serial dilutions onto 293 cells. Presence of virus in the blood was assessed as cytopathic effect on the 293 cells and titer of circulating virus determined. **B.** 3 days later, livers were recovered and used for preparation of cDNA, which was subsequently screened by PCR for levels of mRNA of E1A.

7A

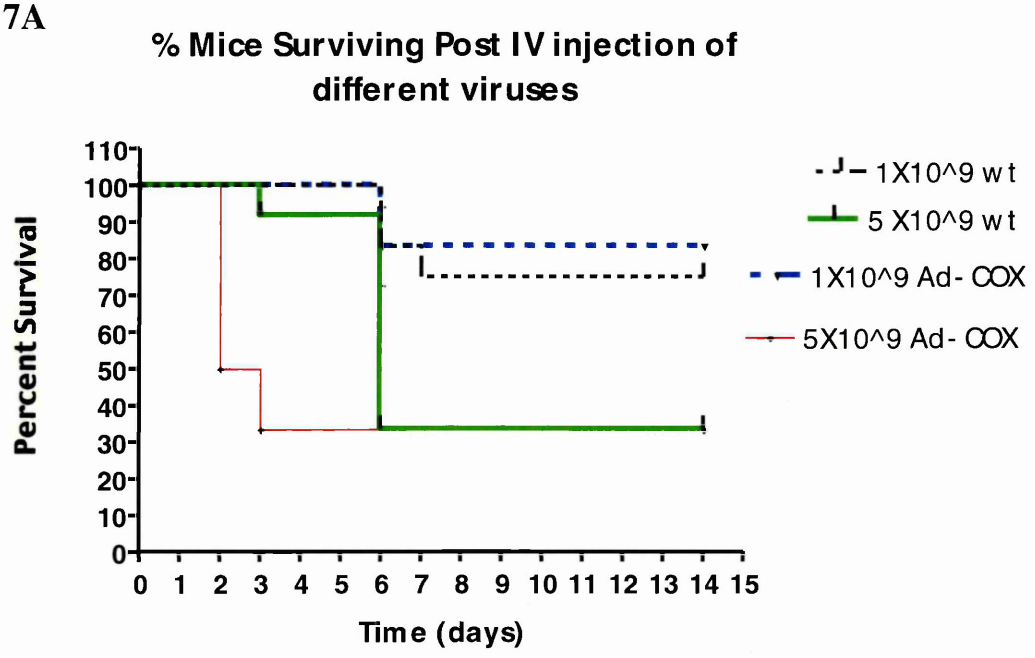


Figure 7A. i.v dosing studies of Ad-E1A-COX and wt Ad-5 administered in C57BL/6J mice. *Data points,* percent of mice surviving following tail vain injection with Ad-E1A-COX or wt Ad-5. mice were euthanized immediately if they show signs of acute toxicities such as anorexia or lethargy. Surviving mice were euthanized 14 days after virus injection.

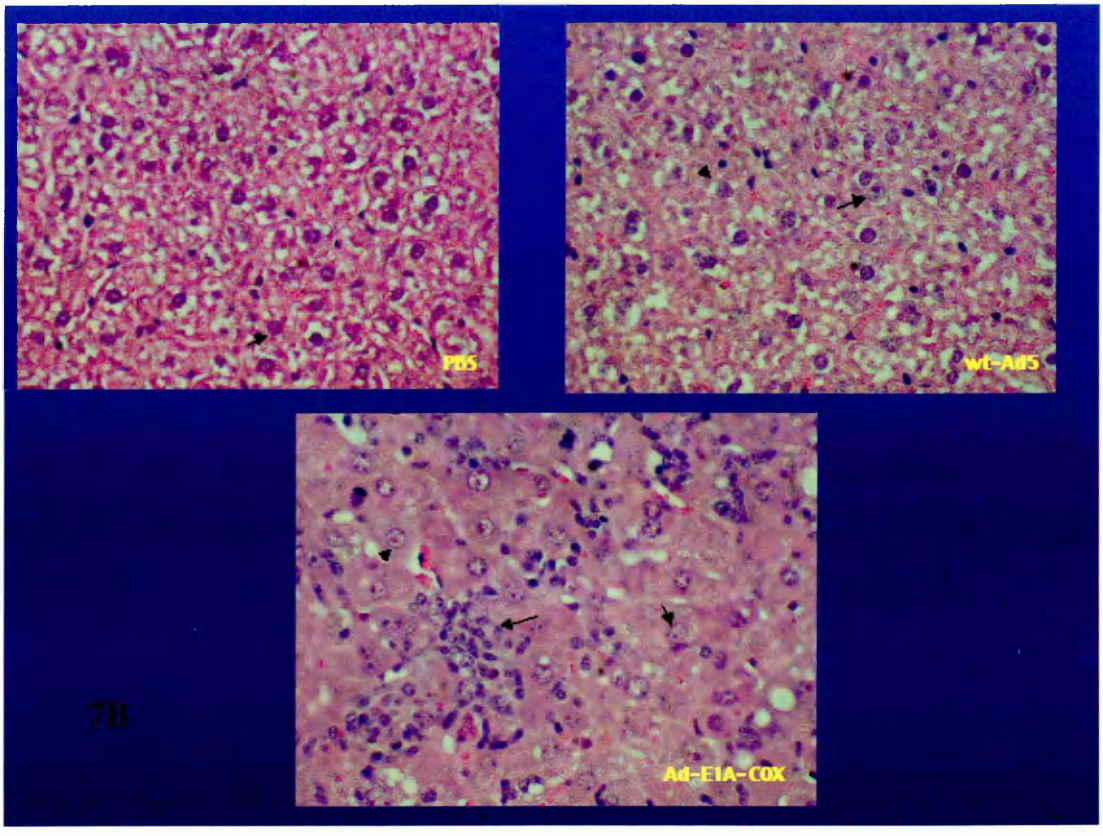


Figure 7B. Liver histology in C57BL/6J mice following a single i.v. injection of Ad-E1A-COX virus. Mice were injected with $5X10^9$ pfu of different viruses. Representative photomicrographs of H&E-stained liver sections from mice died acutely (3-6 days after injection). Arrow showing the immune infiltrates in the liver of the mice treated with Ad-E1A-COX virus.

7C

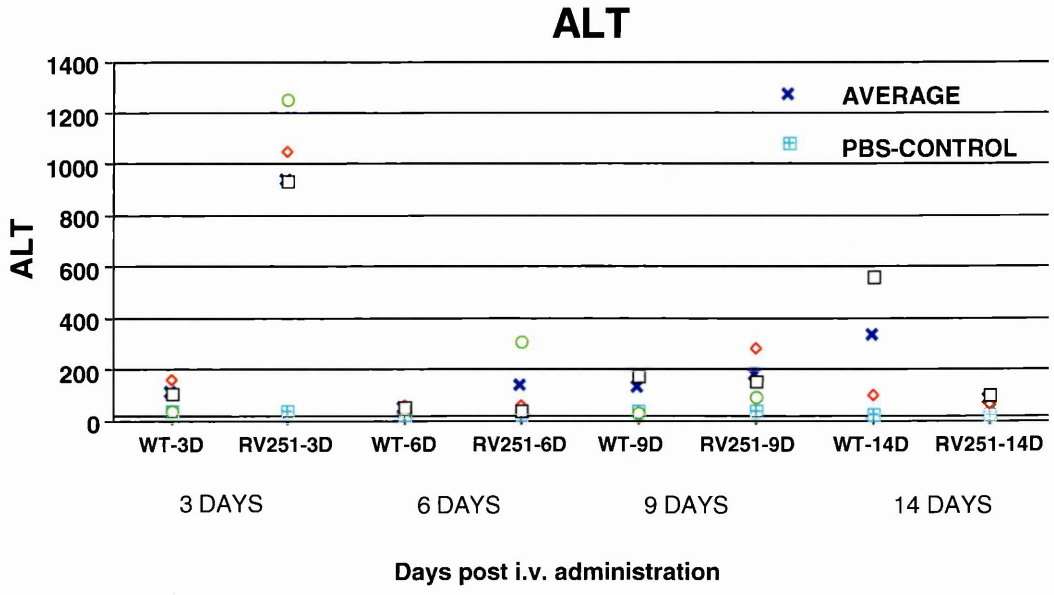
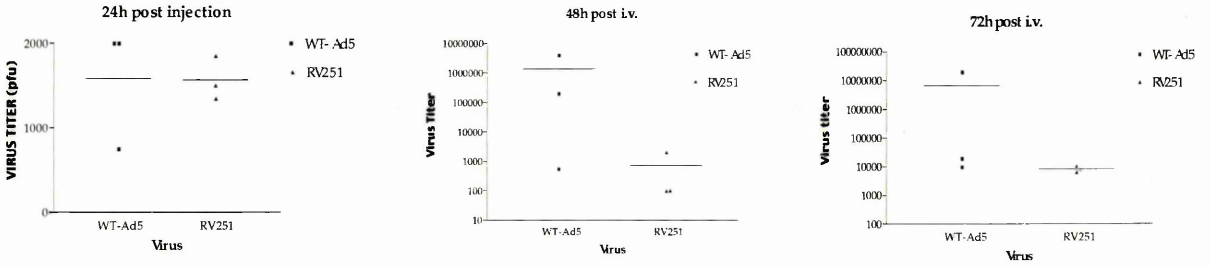


Figure 7C. Animals were treated with PBS of $5X10^9$ pfu of the indicated virus. Serum was collected for every three days interval for 14 days. Each data point represented individual mice.

7D



7E

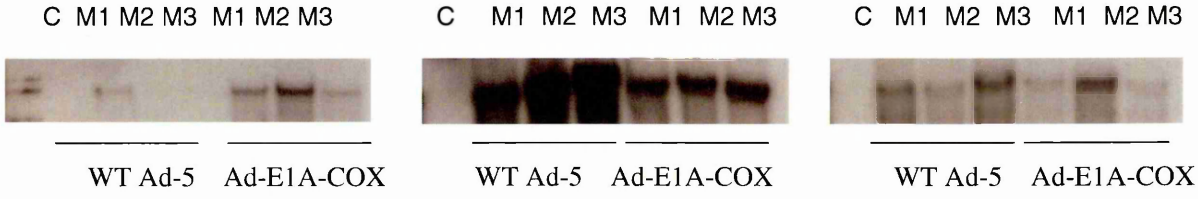


Figure 7D. Virus titer in liver in C57BL/6J following a single i.v. injection of Ad-E1A-COX (RV251) or wt Ad-5 virus. **E.** Southern blot analysis of mouse liver total DNA, 24, 48 and 72 hours following the administration of $5X10^9$ pfu of Ad-E1A-COX or wt Ad-5 virus in C57BL/6J mice. Probing for the 8-kb fragment of adenovirus DNA. Each lane represents individual mice.

Discussion:

In this chapter, we show that tumor cell selective stabilization of mRNA can be used as a novel means to regulate viral gene expression and thus restricting viral replication in the tumor cells. Ligation of the E1A gene to a 469bp region of the 3'UTR of the COX-2 gene (Dixon et al., 2000) is sufficient enough to regulate adenoviral replication to tumor cells expressing activated RAS oncoprotein or, more generally, increased P-MAPK activity. Activated RAS and/or receptor tyrosin kinase signaling, resulting in elevated P-MAPK activity, is characteristic of a very wide variety of human tumor types. The up-regulation of COX-2 is a downstream effect of RAS-mediated transformation (Sheng et al., 2001; Sheng et al., 1998b). Although RAS-mediated overexpression of COX-2 is also associated with increased transcription of the COX-2 gene, a large component of its up-regulation is mediated by selective stabilization of the mRNA of the COX-2 gene in RAS-transformed cells (Dixon et al., 2000; Sheng et al., 2001). Given the clear association in the literature between the proliferative response of cancer cells, P-MAPK activation and regulation of gene expression through selective stabilization of mRNA, we hypothesized that we might be able to use tumor cell selective stabilization of mRNA as a novel means to control therapeutic gene expression in viral vectors for cancer gene therapy. We describe here the construction of a conditionally replication competent adenoviral vector, in which expression of the essential E1A gene is regulated by ligation to the 3'UTR of the COX-2 gene, allowing RAS/P-MAPK-specific stabilization of the mRNA. This is the first description of a replicating adenovirus whose tumor selectivity is based upon control of gene expression at the level of mRNA stability. This strategy has great potential for

expansion since there are many different genes whose 3'UTRs control selective mRNA stability under different physiological, pathological and tumor-associated conditions.

The data presented in this chapter demonstrates that inserting the COX-2 3'UTR element downstream of the E1A gene in the adenoviral genome can restrict viral replication selectively in the tumor cells. Using a panel of human tumor cell lines with different levels of MAPK signaling, we are able to show that the Ad-E1A-COX virus is preferentially oncolytic *in vitro* in human tumor cells with high levels of P-MAPK activity. *In vivo*, the Ad-E1A-COX virus is at least as effective oncolytically as the wild type virus in high P-MAPK expressing tumors (U87 and U251), but generates no significant therapeutic effects in low P-MAPK expressing tumors (U118). It is not yet clear exactly what levels of P-MAPK activity are required for sufficient stabilization of the E1A-COX-2 mRNA to support viral replication in order to achieve therapeutic efficacy. Such information will be important in order to identify the types of cancer that can be targeted by the Ad-E1A-COX oncolytic vectors.

Although adenoviral replication in murine cells is greatly reduced as compared to human cells, different murine models have been extensively used to evaluate the safety and biodistribution of recombinant adenoviral vectors after systemic administration. Previous *in vivo* toxicity studies have shown that the intravenous administration of adenoviral vectors results mostly in hepatocyte transduction (Fechner et al., 1999). We first used the immunocompromised nude murine model to study the toxicity and biodistribution of the Ad-E1A-COX virus after systemic administration. Following intravenous injection of adenovirus, virus could be detected in the blood of the animals receiving Ad-E1A, but not Ad-E1A-COX virus. Moreover, the livers of injected mice expressed appreciable levels of

E1A from the Ad-E1A virus. In contrast, undetectable amount of E1A mRNA could be found in the livers of mice that received similar doses of Ad-E1A-COX. Given the particular sensitivity of the liver as a potential site of toxicity following therapy with adenoviral vectors, this data shows that the presence of the COX-2 3'UTR is sufficient to reduce significantly the levels of E1A expression in nude mice - liver tissue. This would be expected to translate into significantly reduced levels of toxicity should such vectors become disseminated through the circulation.

A majority of patients dying to cancer do so because of metastasis diseases. The treatment of patients with systemic metastasis remains a difficult challenge. One of the goals for any new cancer gene therapy strategy is to target systemic metastasis for more effective and selective treatment against cancer. In the clinical setting, patient's immune system is a major obstacle for any systemic gene therapy. Host immune system eliminates most of the therapeutic viral vectors after systemic administrations, which not only reduces the therapeutic efficacy, but also is the main cause of systemic toxicity induced by the viral vector. Both the systemic metastasis and the host immune system have proven to be the major challenges for the development of successful gene therapy for cancer treatment. So, the safety and therapeutic activity after the systemic administration of any new viral vector for the cancer gene therapy needs to be tested very thoroughly. For this purpose, an evaluation of the toxicity and biodistribution of the Ad-E1A-COX virus were carried out in the presence of the immune system by delivering the virus intravenously in the immunocompetent C57/B6 mice. In this murine model, the dosing regimen of $2.5X10^9$ pfu resulted in 50% lethality (LD50) for both the Ad-E1A COX and the wt Ad-5 virus. Although there was no difference in the LD50 value for both of the viruses, the mice

treated with the Ad-E1A-COX virus started to die much earlier (5 days earlier) than the mice treated with wt Ad-5 virus (**Figure 7A**). The mice that died acutely after systemic Ad-E1A-COX virus administration showed signs of hepatic toxicity including gross pathological changes (liver tissue looked more yellow), histophathological changes and elevated liver enzymes in the serum.

The reported LD50 for the conditionally replicating ONYX-015 in the immunocompromised nude mice is about $5X10^9$ pfu (Heise et al., 1999a), which is two fold higher than the LD50 of the Ad-E1A-COX virus measured in the immunocompetent host. The anti-vector host immuoresponse associated with the liver toxicity in nude mice is very different and probably much less intense than the immunocompetent C57B/6 mice, which is probably due to the fact that nude mice do not have all the necessary immune components. After systemic administration of the Ad-E1A-COX and Ad-E1A viruses in nude mice, we only measured the presence of the E1A mRNA by RT-PCR in the liver tissues and we were not able to detect any E1A mRNA in the mice treated with Ad-E1A-COX virus (**Figure 6A & 6B**). These mice also show no signs of systemic toxicity. So, we think that the discrepancy between the toxicity results in the immunocompromised and immunocompetent murine model is mainly due to the difference in the immune system of these two models.

Another possible way to explain the toxicity and the early onset of mortality by the recombinant Ad-E1A-COX virus is the absence of the immunoregulatory gene E3 in the viral vector. The adenoviral E3 gene products are the most important genes that suppress the host immune response against the virus, which regulates many aspects of the host immune system and creates an environment ideal for viral replication. Ad E3 gene products

such as gp19K block the transport of MHC class I major from the ER to the plasma membrane and downregulate CTL response. Another E3 gene product, the 10.4K/14.5K protein, down-regulates death receptor and its ligand Fas and TRAIL by receptor internalization and inhibits TNF-induced cytolysis and production of chemokines by blocking NF-kB signal transduction. Most of the first generation adenoviral vectors are E3 deleted because E3 is not necessary for viral replication and also to create some space for the transgene. So the early onset of the toxicity induced by the Ad-E1A-COX virus may be due to the absence of the E3 genes. In contrast, the control wt Ad-5 virus may be able to suppress the early toxicity induced by the immune system because of the intact E3 gene in the viral genome. So, it is probably not fair to compare the toxicity between these two viruses due to the difference in the E3 gene. A proper way to evaluate the toxicity of the AdE1A-COX virus would be a virus with the same first generation viral backbone as AdE1A-COX virus, but without any COX-2 3'UTR.

We also cannot exclude the possibility that the Ad-E1A-COX virus may replicate in some specific organ non-selectively than the wt Ad-5 and may induce the toxicity in the immunocompetant model. There are anatomical areas, such as the kidney, the central nervous system and the seminal vesicles, where COX-2 expression is elevated compared to other organs and may support Ad-E1A-COX replication. But there was no sign of replication for both viruses in major organs such as heart, lung and the kidney. Liver was the only organ where we able to detect some non-specific Ad-E1A-COX virus replication. Mice treated with either of the viruses showed increased amounts of viral titer and viral DNA measured in the liver. But the replication kinetics of these two viruses in the liver was very different. Compared to wt As-5 virus, the Ad-E1A-COX virus showed an increase in

viral DNA synthesis at 24 hours post injection (**Figure 7E**), but there was no difference between the viral titer at this time point (**Figure 7D**). We do not know why at 24 hours post injection, the recovered viral DNA was higher in the liver of the mice treated with Ad-E1A-COX virus than the wild type Ad-5 virus or why this increased Ad-E1A-COX viral DNA in the liver do not correlate with the recovered viral titer at this time point. One possibility may be that at 24h post administration, the increased viral DNA synthesis in the liver of the mice treated with Ad-E1A-COX virus is due to the higher replication of that virus, but the titer is not changed because of the inability of this virus to control the host innate immune response, which also may have cleaned up the virus and resulted in more intense hepatotoxicity. Together, these data confirm that there is an early onset of liver toxicity after systemic administration of recombinant Ad-E1A-COX virus compared to wt Ad-5 virus in the immunocompetent murine model.

Host immune response is a major obstacle to the systemic administration of recombinant adenovirus. Ad vector induced acute inflammation not only reduces the gene transfer efficiency and vector persistence, but also causes profound damage to normal tissue and significant morbidity in the transduced hosts (Raper et al., 2002). The specific mechanisms underlying the acute immune response against the Ad viral vector are not well understood. Both viral entry and the adenoviral gene products induce proinflammatory cytokines and chemokines cascade that have harmful effects. During viral entry adenovirus fiber proteins interact with cellular Coxsackie adenovirus receptor (CAR) (Bewley et al., 1999) and the RGD motifs of the penton base interact with the $\alpha_v\beta_3$ and the $\alpha_v\beta_5$ integrins (Li et al., 2001a). These interactions activate some key intercellular signaling pathways, which are critical for viral endocytosis (Li et al., 1998) and facilitate viral particles transport to the

nucleus by altering the actin cytoskeleton (Suomalainen et al., 1999). But the host also uses the vector activated intercellular signaling pathways as the alarm signals for viral infection and induces innate immune responses, the first line of host defense against any viral infection. The rapid and potent innate immune reactions induced by the adenoviral vectors can have devastating consequences as seen in the clinical trial of gene therapy for ornithine transcarbamylase (OTC) deficiency (Raper et al., 2002).

In vitro and *in vivo* studies have demonstrated that during cell entry, the adenovirus activates the intercellular MAPK-signaling cascade for the efficient transport of viral particles to the nucleus. The host cells also use the same signaling pathway to trigger early inflammation and subsequent antiviral immunity. Within 10 min of Ad-LacZ transduction, MAPK dependent IL-8 induction was observed in HeLa cells. Similar studies have showed that the activation of p38 MAPK within minutes after adenovirus entry in epithelial cells (Suomalainen et al., 1999). A chemical inhibitor against the p38 MAPK signaling pathway blocked Ad vector induced chemokine IP-10 expression, providing a link between early vector-induced signaling and proinflammatory gene expression (Tibbles et al., 2002).

MAPK signaling plays an important role in regulating different gene expressions that supports both the viral life cycle and the host immune responses. Once activated, MAPKs can directly activate transcription factors and transcriptional co-regulator via phosphorylation or activate the downstream kinases that can regulate transcription and mRNA stability. Crofford and Roessler showed that the adenovirus treatment of the liver synoviocytes increased levels of COX-2 mRNA and protein (Crofford et al., 2005). Viral gene expression was not required for the early COX-2 induction and inhibition of the p38 MAPK pathways to suppress the COX-2 expression.

During the systemic administration of the recombinant viral vector, the host cannot distinguish between the infectious virus and the therapeutic virus and therefore inducing potent immune responses. Also, the immune responses generated by the host against the recombinant adenoviral vectors are more intense than the wild type virus infection because of the deletion of E3 immunosuppressive genes. One possibility is that the MAPK signaling cascade which is activated during the vector entry process through the interaction with the host $\alpha_v\beta_3$ and the $\alpha_v\beta_5$ integrins also creates an environment to stabilize COX-2 mRNA as a part of the host immune response. Here, we used the COX-2 3'UTR to selectively stabilize the mRNA of the adenoviral replicative essential E1A gene in the RAS transformed environment and thus controlled viral replication in the tumor cells. Inflammatory signaling cascades like MAPK, that get activated during the viral entry process can also induce inflammatory molecules like COX-2 or the adenoviral gene E1A that is fused with the COX-2 3' UTR by stabilizing the mRNA via the AU-rich sequences in the 3'UTR. This may lead to the nonspecific stabilization of E1A mRNA in any cells or tissues that contain viral entry receptors and are capable of responding to viral infection. So the enhanced toxicity observed by the Ad-E1A-COX after the systemic administration in the immunocompetent model may be due to an inflammatory environment induced by the viral vector in the target organ such as liver, which then stabilizes E1A mRNA, supporting nonspecific virus replication. Also in the immunocompetent animal, the host immune response is more intense due to the presence of all the necessary immune components, which creates a much more favorable condition to stabilize the mRNA of the proinflammatory genes like COX-2 or E1A fused with the COX-2 3'UTR.

Many different genes with the AU-rich 3'UTR confer destabilizing activity on their cognate mRNA but their actions are reversed under certain physiological conditions. These include hypoxia responsive 3'UTR (Claffey et al., 1998) (Maity & Solomon, 2000), radiation responsive elements and 3' UTR which increased mRNA stability in proliferating cells (Lee et al., 1998; Maity et al., 1997). In this report, we were able to show that it is possible to control transgene expression and viral vector replication by using tumor cell selective mRNA stabilization via the COX-2 3'UTR. It will be important to choose these elements thoughtfully in order to develop a conditionally replication viral vector. During inflammation and cellular transformation, the expression of many inflammatory genes is controlled at the level of their mRNA stability. These genes will probably not be the best candidates for targeting tumor through their 3' UTR.

To our knowledge, this is the first time that conditionality of adenoviral replication has been conferred solely at the post-transcriptional level of control of E1A expression. However, based on previous experience with designing tumor cell specific vectors where initially unpredicted results can surface upon broader testing, it seems likely that there will be apparently suitable tumors (i.e. mutated RAS or elevated P-MAPK activity) in which the level of control conferred by the 3'UTR is insufficient to allow completely controlled levels of replication. Additionally, there are likely to be anatomical sites, physiological conditions and inflammatory environments which are amenable to stabilization of the COX-2 3'UTR region (Cao & Prescott, 2002), thereby allowing viral replication at extra-tumoral sites where COX-2 is normally induced (Turini & DuBois, 2002). Therefore, we envisage that the 3'UTR strategy will be most effective in the context of a mosaics of regulatory elements combined to confer multiple levels of specificity to the resultant virus.

Other targeting strategies such as transcriptional regulation of the E1A gene can very readily be combined with the COX-2 3'UTR (indeed the COX-2 promoter has itself been used to target recombinant vectors (Cao & Prescott, 2002)), as can incorporation of molecular features that target tumor cell specific mutations, such as loss of p53 or downstream effectors. Thus, multi-component targeting will be most effective at generating truly tumor selective vectors, which can be delivered to extra-tumoral sites without allowing unacceptable levels of viral replication. Based on our studies here, we propose that mRNA (de-) stabilizing elements be considered as one of the component cassettes of such mosaically regulated viruses.

The data presented in this chapter from part of the following paper:

Ahmed A, Thompson J, Emiliusen L, Murphy S, Beauchamp DR, Suzuki K, Alemany R, Harrington K and Richard G. Vile. A Conditionally Replicating Adenovirus Targeted to Tumor Cells Through Activated RAS/P- MAPK-Selective mRNA Stabilization. *Nature Biotech*; 2003 Jul; 21(7): 771-7.

Chapter 5

Retargeted Intratumoral Expression of a Fusogenic Membrane Glycoprotein by Tumor Cell Selective mRNA Stabilization Enhances the Efficacy of Replicating Adenovirus Therapy.

5.1 Introduction

This chapter describes a novel approach to enhance and retarget the oncolytic potency of adenovirus vector by combining the ability of the COX-2 3'UTR to stabilize mRNA and regulate gene expression specifically in cells with elevated levels of activated Ras/MAPK with a viral Fusogenic Membrane Glycoproteins (FMG), which induces tumor cell killing through induction of fusion of tumor cells to form large multinucleated syncytia (Fielding et al., 2000) (Bateman et al., 2000b). In the previous two chapters, we show that the 469 bp COX-2 3'UTR is able to regulate the expression of a viral gene, thus controlling viral replication and oncolysis. Here we demonstrate that the FMG mediated fusion and tumor cell killing can also be control by the COX-2 3'UTR element and this FMG-induced tumor selective syncytia is able to enhance the oncolytic potency of replication competent adenoviral vector both in vitro and in vivo.

The use of conditionally replication-competent viruses for the treatment of cancer has been more prominently considered due to the problems associated with poor intratumoral spread and low transduction efficiency in vivo (Vile et al., 2002). In order to achieve clinically relevant therapeutic efficacy, it is essential to improve spread of the therapeutic virus within tumors where other cell types, cell matrix, and areas of necrosis exist and viral vector tends to be trapped in between the sub-cellular compartment (Sauthoff et al., 2003). Many different strategies used to increase efficacy of adenovirus include engineering viral genes to enhance virus release and spread, and aiming viruses with additional therapeutic genes that might either enhance viral cytotoxicity and/or provide additional therapeutic benefits (Ramachandra et al., 2001) (Heise et al., 2000). However, poor intratumoral spread

remains an important hurdles that need to be overcome before replicating adenoviral vector can be used efficiently even against the localized tumor burdens (Vile et al., 2002).

Previously, several laboratories including ours have shown that tumor cell killing can be obtained by gene transfer of the FMG and the FMG-induced tumor cell killing also create a immunostimulatory environment, which can enhance the therapeutic efficacy (Bateman et al., 2000b) (Higuchi et al., 2000). Moreover, a recent report was able to demonstrate that the HIV gp120 FMG induced fusion facilitates the spread of adenoviral vectors through a monolayer of tumor cells and increases viral release from the infected cells (Li et al., 2001b). Based on these reports, we therefore explored the use of FMG in combination with the COX-2 3'UTR to selectively enhance the therapeutic efficacy of the oncolytic adenoviral vector. The data presented in this chapter demonstrates that by using the COX-2 3'UTR mediated, FMG-induced retargeted tumor cell fusion in tandem with the oncolytic properties of replicating adenovirus enhance the therapeutic efficacy of both approaches synergistically by increasing the release of viral particles from infected cells that are fused by FMG and/or the spread of viral particles through the tumor via the syncytia.

5.2 Results

5.2.1 Co-transduction of human tumor cells with sub-therapeutic doses of plasmid DNA expressing an FMG and replicating adenovirus leads to extensive tumor cell killing *in vitro*

Transfection of 10^6 confluent tumor cells from various different lines (Mel624 or Mel888, human melanoma; LnCap human prostate carcinoma; U118 or U251 glioma) *in vitro* with 0.5μg of plamsid DNA expressing the hyperfusogenic Gibbon Ape Leukemia Virus

(GALV) FMG (Fielding et al., 2000) (Bateman et al., 2000a) (pCR3.1-GALV) routinely led to over 90% of the cells being killed 96 hours following transfection. Similarly, infection of these cell lines with wild type adenovirus serotype 5 (Ad5-Wt) at an m.o.i. of 0.1 led to cytopathicity of over 95% of cells (**Figure 1A**). To study the interaction of FMG expression with Ad5-Wt infection, we selected treatment doses (0.01μg plasmid DNA and 0.001 m.o.i of Ad5-Wt) at which less than 10% of the tumor cell cultures were killed by either treatment alone (**Figure 1B**). However, co-transduction with these suboptimal doses of both pCR3.1-GALV (0.01μg) and Ad5-Wt (m.o.i. 0.001) induced significantly increased cytotoxicity compared to either treatment alone (>95% cell killing) (**Figure 1B**).

5.2.2 Suboptimal doses of plasmid FMG DNA and replicating adenovirus co-operate to eradicate small-established tumors.

To investigate whether these *in vitro* results also have relevance to *in vivo* virotherapy, we first used direct intratumoral injection of suboptimal doses of Ad5-Wt or pCR3.1-GALV into very small established (0.2cm diameter) human tumor xenografts. Three intratumoral injections of low doses of either pCR3.1-GALV (0.5μg/injection) or Ad5-Wt virus at a low dose ($3x10^7$ pfu/injection) had no significant effect on tumor cure (**Figure 3**). However, simultaneous co-transduction of tumors with replicating adenovirus stocks and syncytia-inducing GALV plasmid DNA led to regression of all tumors under these conditions (**Figure 3**) such that, at the end of the experiments shown in **Figure 3 A** and **B**, 10 of 10 mice were tumor free in the (pGALV + Ad5-Wt) treatment groups.

We confirmed that the therapeutic effects of pCR3.1-GALV and Ad5-Wt co-transduction were dependent upon expression of the GALV FMG by including an additional treatment group in the experiments of **Figure 3**. In previous two chapter, we have reported that gene

expression can be effectively targeted to cells expressing an activated mutant RAS oncogene and/or high levels of P-MAPK by ligation of a therapeutic gene to the 3'Untranslated Region of the Cyclooxygenase 2 gene (COX2-3'UTR). The COX2-3'UTR efficiently destabilizes mRNA molecules in cells with low P-MAPK activity but selectively restabilises the mRNA molecules and, therefore, protein expression in cells with high P-MAPK activity. We constructed an expression plasmid, pCR3.1-GALV-COX (**Figure 2**) in which 469bp of the COX-2 3'UTR destabilizes expression of the GALV FMG in low P-MAPK expressing cells (such as the U118 glioma) but selectively restabilises expression in high P-MAPK expressing cells (such as U251). Cotransduction of small established U251 tumor xenografts with Ad5-Wt and pCR3.1-GALV-COX was as effective therapeutically as treatment with Ad5-Wt and pCR3.1-GALV (10 of 10 mice tumor free at the end of the experiment in **Figure 3B**); however, treatment of the low P-MAPK tumor U118 with Ad5-Wt and pCR3.1-GALV-COX, in which GALV expression is significantly reduced by COX-2 3'UTR-mediated destabilization of the mRNA, was no more effective than treatment with Ad5-Wt alone (**Figure 3A**). These results confirm that GALV expression can be targeted to tumor cells overexpressing P-MAPK and indicate that the therapeutic effects observed with co-transduction of tumors with Ad5-Wt and GALV expressing plasmids are dependent upon expression of the FMG. To confirm the *in vitro* findings of enhanced viral spread through co-expression of the pGALV vector, the experiment of **Figure 3A** was repeated except that the tumors were resected two days following injection. Tumor cells were dissociated *in vitro* and analyzed by FACS for expression of the viral E1A protein. As can be seen from **Figure 3C**, even at early times after transduction *in vivo,* significantly enhanced numbers of infected cells were seen in tumors co-transduced

with replicating virus and pGALV (~8% of the dissociated cells) compared to the virus alone (~1%).

5.2.3 Syncytial formation enhances spread of adenoviral vector through a monloayer

The increased potency of GALV transfection in combination with infection by replicating virus might be explained simply by enhanced transfection efficiency of tumor cells in the presence of lysosomal disrupting adenoviral infection. To address this possibility, we measured GFP expression 48 hours following transfection in cultures transfected with pCR3.1-GFP plasmid DNA (0.01μg) in the presence or absence of co-infecting Ad5-Wt (moi 0.001). Although results differed slightly for each cell line, the presence of Ad5-Wt (at these low m.o.i) increased the transfection efficiency with plasmid DNA by, at most, about two fold (typically increasing the number of GFP positive cells from 4% to 6-7% in the case of Mel624 cells) (**Figure 4A**). However, transfection with at least 50 fold more plasmid DNA was required to generate levels of cytotoxicity by GALV alone comparable to those seen in the combination of low dose pCR3.1-GALV and Ad5-Wt (**Figure 1A**) suggesting that enhanced transfection efficiency alone was not sufficient to explain the co-operation between GALV transfection and Ad5-Wt infection.

It has recently been reported that syncytial formation enhances the dispersion of adenoviral particles through a monolayer *in vitro* (Li et al., 2001b). Therefore, we examined the ability of a replicating adenovirus to mobilize a replication-incompetent Ad-GFP vector from human tumor cells into murine B16 melanoma cells in co-culture. Human tumor cells are highly susceptible to GALV-mediated cell fusion but B16 murine melanoma cells do not express the receptor for GALV and so will not fuse to GALV-expressing cells. In the experiment described in **Figure 4B**, the only way that the GFP reporter gene would

transduce B16 cells in the mixed cultures would be through mobilization of Ad-GFP from the human tumor cells by the replication competent Ad5-Wt virus. The number of GFP-transduced B16 cells at the end of the experiment was assessed by FACS analysis. The presence of ongoing cell fusion within cultures of human tumor cells significantly enhanced mobilization of Ad-GFP for infection of bystander B16 cells when replicating Ad5-Wt was present (**Figure 4B**).

5.2.4 Syncytial cultures produce increased levels of viral titer.

The increased mobilization of the Ad-GFP vector (**Figure 4B**) in the presence of syncytia may be due to an increase in the viral titer released on a per cell basis, an increase in the dispersal of the same amount of virus through the culture (Li et al., 2001b), or both. The titer of cell free virus released into the supernatants from the cultures of **Figure 4B** were significantly higher in the presence of syncytia relative to the same number of cells in non-fusing cultures ($p<0.03$ for U87 cells and $p<0.01$ for U118 cells) (**Table 1**). The difference in the amount of cell-associated virus (recovered from the cell pellets) was less marked between fusing and non-fusing cells but was still significantly enhanced from fusing cells ($p<0.05$ for U87 cells and $p<.0.01$ for U118 cells) (**Table 1**). The increase in cell free virus may in part be due to the increased lysis of the human tumor cells by FMG-mediated cell killing and release of cell associated virus. These data show that presence of fusing cells increases both the total amount of virus produced and increases the amount of virus released into the culture supernatant, which at least partly will explain the more efficient spread of adenovirus through the culture (**Figure 4B**) (Li et al., 2001b).

5.2.5 Increased titer associated with syncytia occurs through posttranscriptional upregulation of E1A expression.

RtPCR analysis indicated that the increased viral titers described in **Table 1** were not due to greater levels of transcription of the viral E1A gene within Ad5-Wt-infected, fusing human tumor cell cultures (**Figure 5A**), data confirmed by Northern blotting (not shown). In contrast, levels of E1A protein were significantly enhanced (up to 10 fold depending upon the experiment) within fusing, as opposed to non-fusing, tumor cell cultures (**Figure 5B**). These data are consistent with our previous observations (Higuchi et al., 2000), as well as those of others (Mi et al., 2000) that as syncytia develop they become highly metabolically active (Higuchi et al., 2000) and generate high levels of protein production compared to the same number of non-fused cells.

Figure 1A

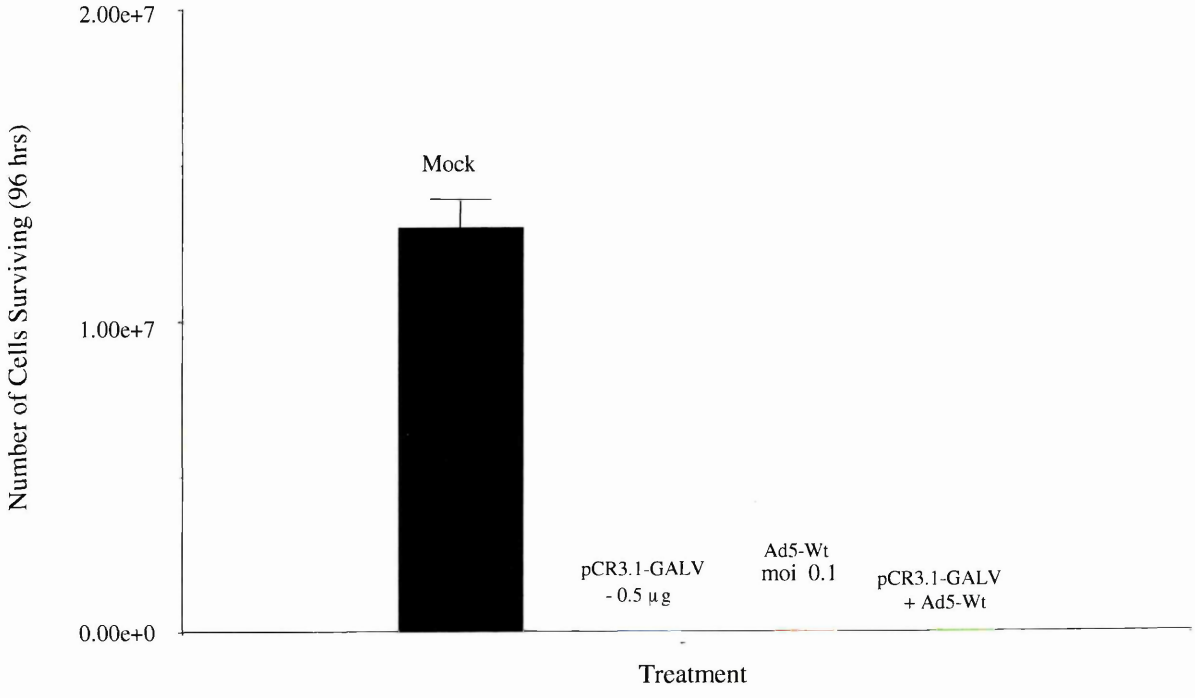


Figure 1B

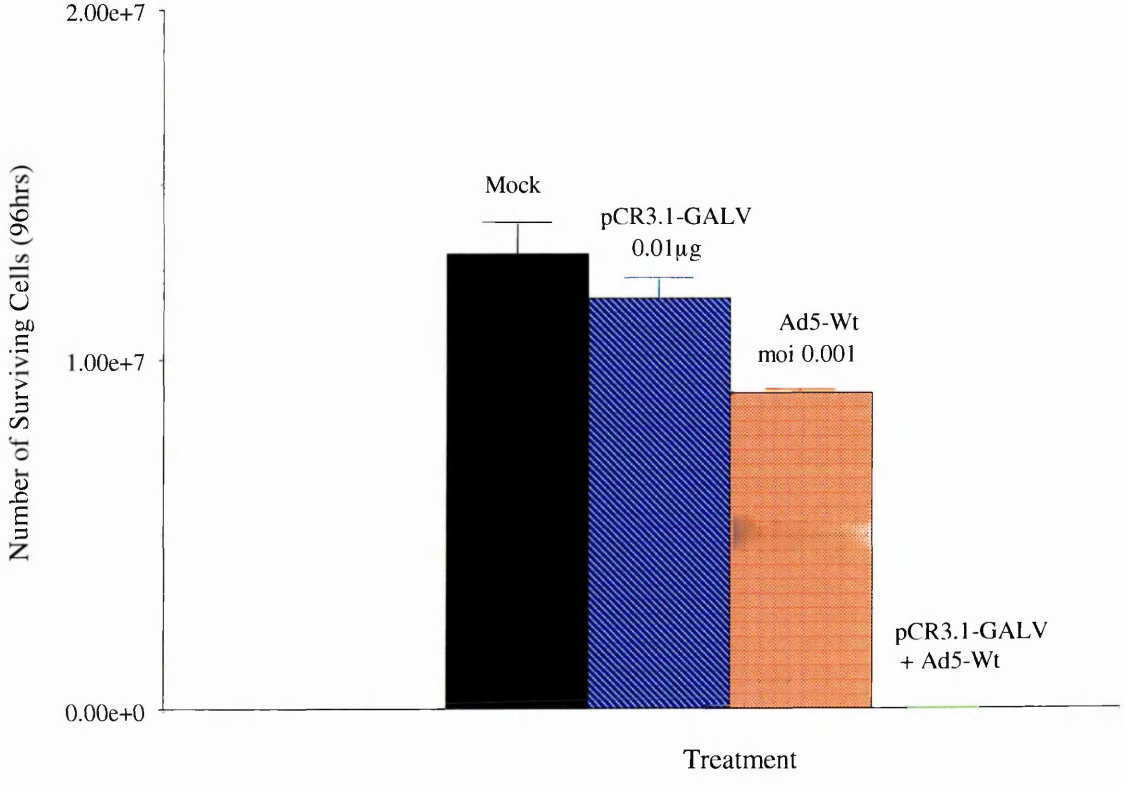


Figure 1. A. 10^6 confluent tumor cells (Mel624 shown here) were either transfected using the Effectene reagent *in vitro* with 0.5μg of plamsid DNA expressing the GALV FMG (pCR3.1-GALV), were infected with wild type adenovirus serotype 5 (Ad5-Wt) at an m.o.i. of 0.1 or were transfected with 0.5μg plasmid DNA admixed with Ad5-Wt (pCR3.1 + Ad5-Wt). 96 hours later, surviving cells were counted. **B.** The same experiment was performed using 0.01μg of plasmid DNA, 0.001 moi of Ad5-Wt or both admixed. With the low dose of plasmid DNA, individual pockets of syncytia were formed but these were too infrequent to cause wide scale fusion of the entire population of cells and were eventually overwhelmed by proliferating tumor cells.

Figure 2

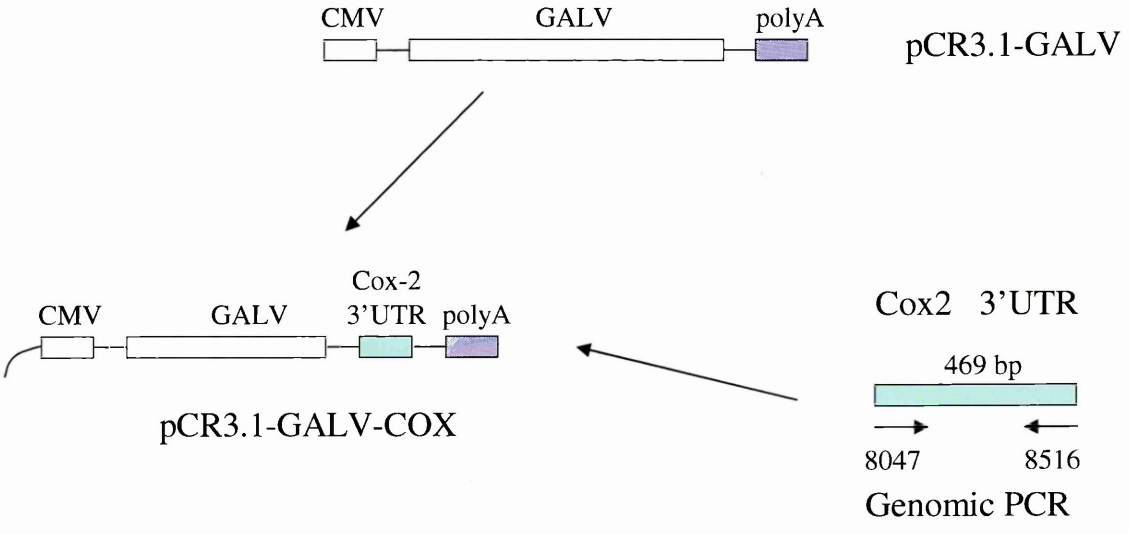


Figure 2. Construction of the CMV-GALV-COX construct.

Figure 3A

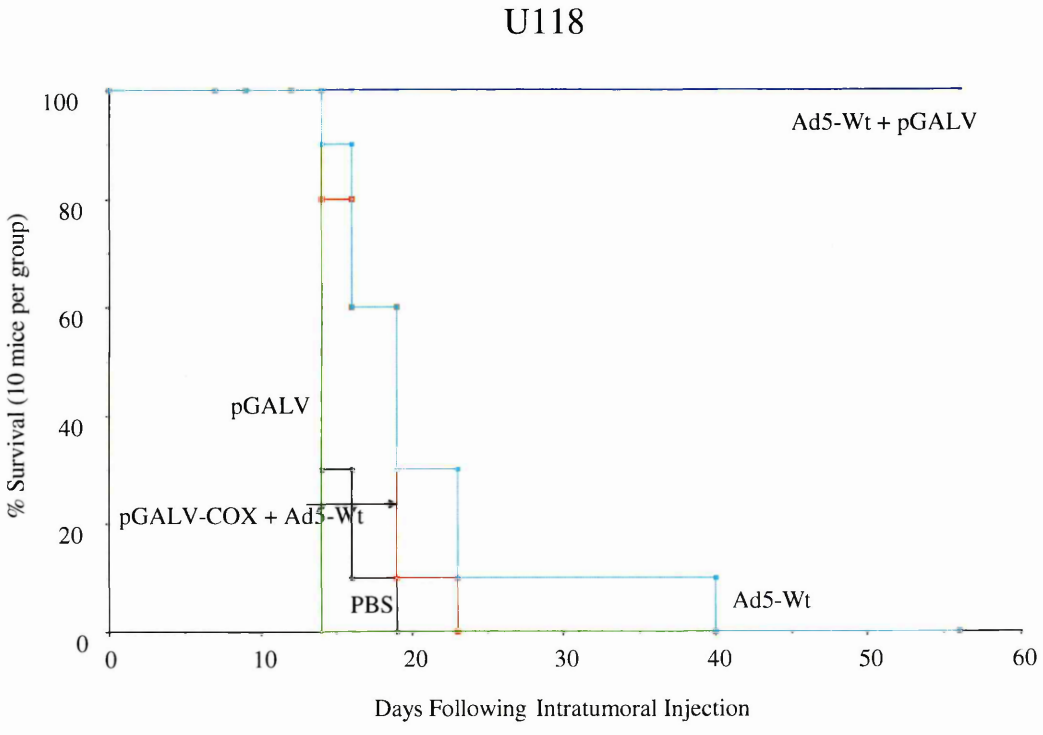


Figure 3B

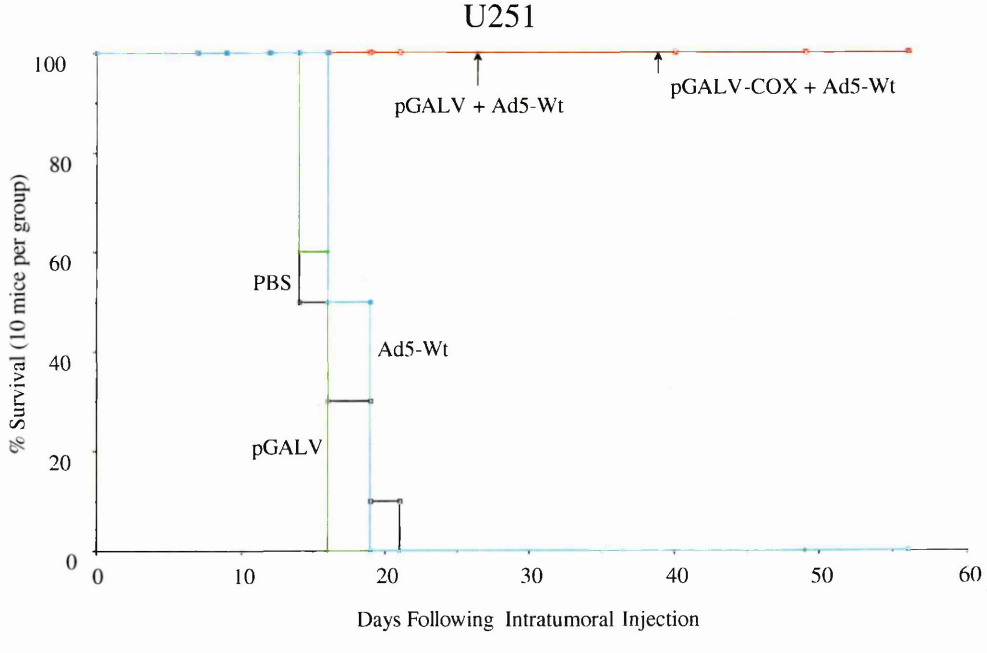


Figure 3. Co-administration of plasmid GALV and Ad5-Wt virus can cure small palpable tumors. $5x10^6$ U118 (**A**) or U251 (**B**) tumor cells were injected subcutaneously in athymic nude mice. When tumors were palpable (~0.2cm diameter) different groups (10 mice per group) were injected with PBS (PBS); or with low dose pCR3.1-GALV (0.5μg/injection) plasmid DNA (pGALV); or with Ad5-Wt virus ($3x10^7$ pfu/injection) (Ad5-Wt); or with pCR3.1-GALV (0.5μg/injection) + Ad5-Wt virus ($3x10^7$ pfu/injection) (pGALV+Ad5-Wt). Another group of tumors was also injected with 0.5μg of the plasmid pCR3.1-GALV-COX (**Figure 2**) along with the Ad5-Wt (pGALV-COX + Ad5-Wt) in which 469 bp of the COX-2 3'UTR is ligated downstream of the GALV cDNA (see text). Tumor growth was monitored for a further 62 days. Animals were sacrificed when tumor size reached 1.0cm in any diameter.

Figure 3C

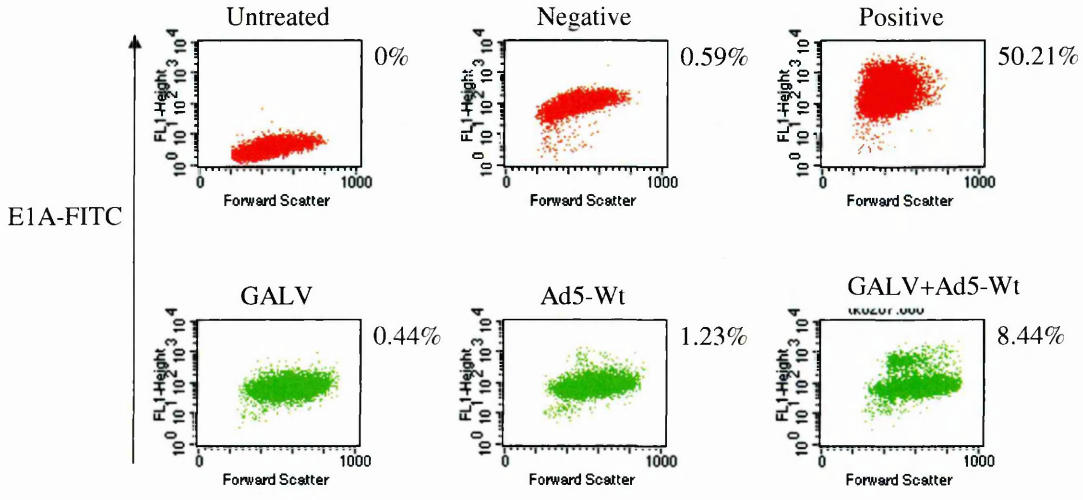


Figure 3C. The experiment of 3 A and B. above was repeated except that the tumors were resected two days following injection with pGALV (GALV), replicating virus (Ad5-wt) or pGALV and Ad5-wt (GALV + Ad5-wt). Tumor cells were dissociated *in vitro* and analyzed by FACS for expression of the viral E1a protein using a rabbit polyclonal antibody against E1a (Santa Cruz Biotech. Santa Cruz, CA) and a FITC labeled anti rabbit IgG. As controls for the FACS analysis, uninfected, cultured U251 cells were treated with no secondary antibody (Untreated) or with both primary and secondary antibodies (Negative). The positive control was U251 cells infected with wild type Ad virus 24 hours previously (Positive).

Figure 4A

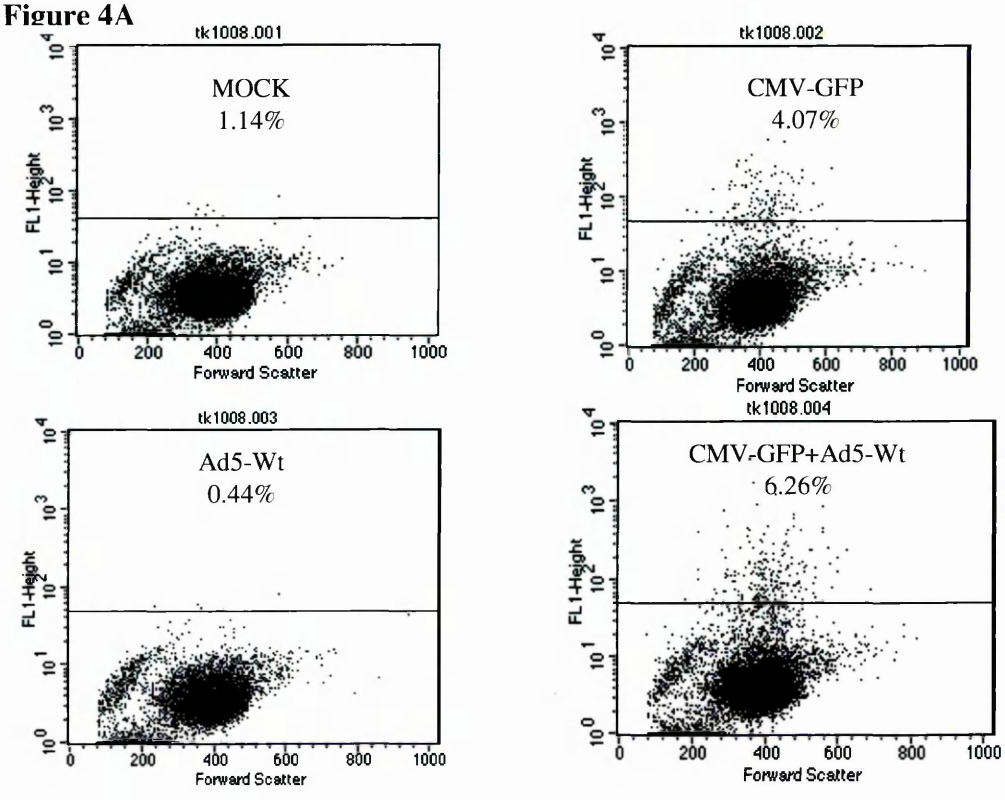


Figure 4B

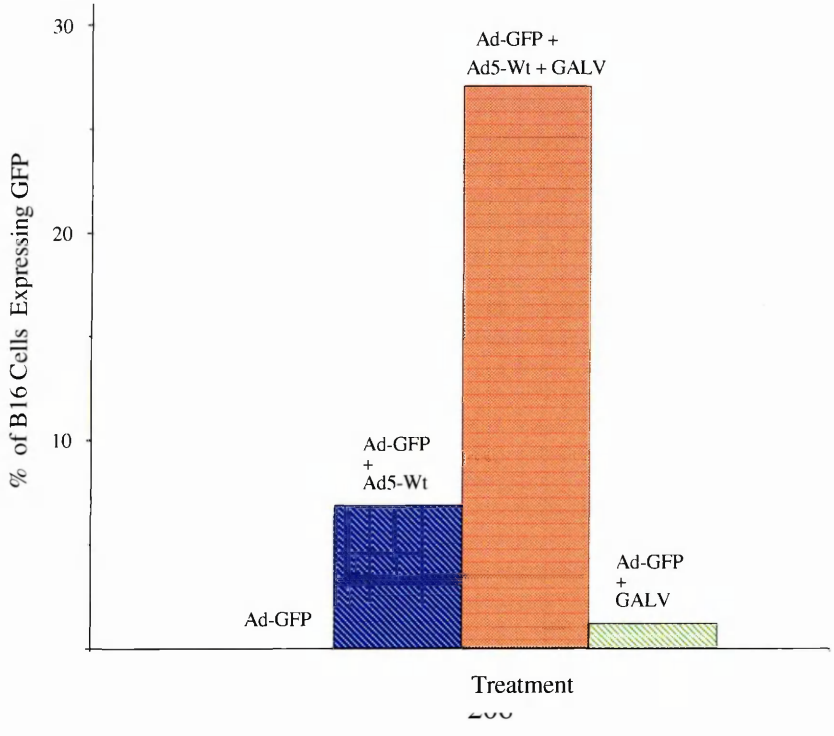


Figure 4. Co-transduction of human tumor cells with a fusogenic membrane glycoprotein and replicating adenovirus increases the spread of virus through the culture. A. GFP expression was measured 48 hours following transfection of Mel624 cultures with pCR3.1-GFP plasmid DNA (0.01μg) (CMV-GFP) in the presence or absence of co-infecting Ad5-Wt (moi 0.001) (Ad5-Wt). **B.** 10^6 cells were transfected with pCR3.1-GALV plasmid DNA (0.01μg) to induce syncytia formation or with or pCR3.1-empty as a control and/or were infected with a mixture of replicating adenoviral stocks (m.o.i. of 0.001) mixed with replication defective Ad-GFP (m.o.i. 0.1). 24 hours later, cultures were washed 3x in PBS and then $5x10^4$ B16 cells, pre-labeled with Cell Tracker Orange dye, were added to the cultures. Since B16 cells will not be incorporated into syncytia, the only way that the GFP reporter gene would transduce these cells would be through mobilization of Ad-GFP from the human tumor cells by the replication competent Ad5-Wt. The number of GFP transduced B16 cells at the end of the experiment (4 days later) was assessed by FACS for double stained (Orange and Green) cells. Similar results were also seen with U118 and U251 cells.

Table 1

(Virus Recovered p.f.u/ml on 293 cells)

Original Transduction Treatment		Cell Free		Cell Associated	
		U87	**U118**	**U87**	**U118**
GALV + Ad5-Wt	+ AdGFP	9×10^4	2×10^5	5×10^6	7×10^6
GALV + ----	+ AdGFP	0	0	0	0
MOCK + Ad5-Wt	+ AdGFP	1×10^3	3×10^3	1×10^6	7×10^5
MOCK + ----	+ AdGFP	0	0	0	0

Table. Syncytial formation is associated with increased viral production. 10^6 U87 (high level of activated MAPK) or U118 (low level of activated MAPK) cells were transfected with pCR3.1-GALV plasmid DNA (0.01μg) to induce syncytia formation or with or pCR3.1-empty as a control and/or were infected with a mixture of replicating adenoviral stocks (m.o.i. of 0.001) mixed with replication defective Ad-GFP (m.o.i. 0.1), cell culture supernatants were recovered and cells were trypsinised, washed in PBS and lysed by three cycles of freeze thawing. Viral titers from the supernatants (Cell Free) and from the cell pellets (Cell Associated) were then determined by a plaque assay on 293 cells as described in material and methods.

Figure 5A

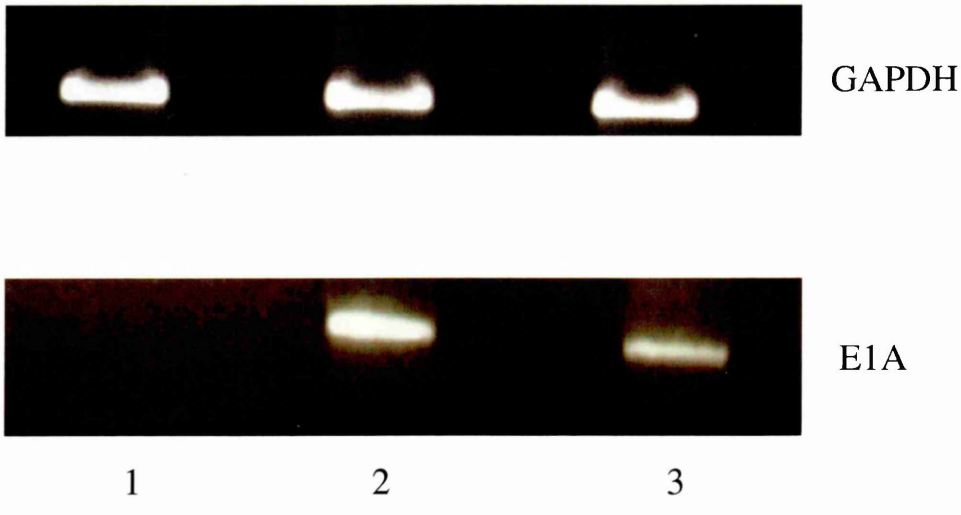


Figure 5B

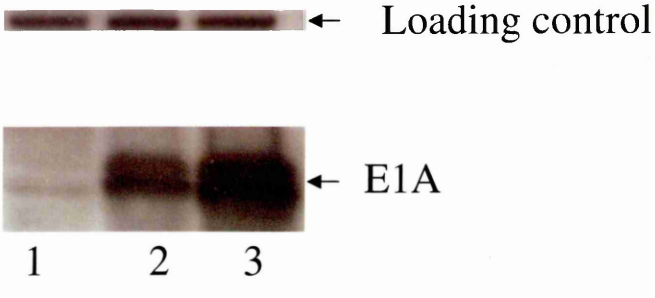


Figure 5. Increased viral titer and spread in syncytial cultures is associated with elevated levels of E1A protein but not mRNA. 10^6 confluent Mel624 tumor cells were either transfected using the Effectene reagent *in vitro* with 0.01μg of plamsid DNA expressing the GALV FMG (pCR3.1-GALV) (lane 1), were infected with wild type adenovirus serotype 5 (Ad5-Wt) at an m.o.i. of 0.001 (lane 2) or were transfected with 0.01μg plasmid DNA admixed with Ad5-Wt (pCR3.1 + Ad5-Wt) (lane 3). 48 hours later RNA was prepared from the cultures and used **A.** for production of cDNA. Primers specific for E1A (5'-TTCCTCAAGAGGCCACTCTTG-3' and 5'-CACGCCATGCAAGTTAAACA-3') were used to assess the amount of E1A message present in the cultures. Equal loading of samples was assessed by riper for GAPDH. **B.** In addition, protein was prepared from cell lysates and Western Blot analysis used to determine the relative levels of expressed E1A protein. Equal loading of samples was confirmed as shown in the upper panel.

5.4 Discussion

Oncolytic adenoviruses are a promising new concept for the treatment of cancer. One of the most attractive advantage of replicating oncolytic viruses is that a single virus can selectively amplify several thousand-fold within a tumor cell and the progeny viruses then have the capacity to spread and infect uninfected tumor cells, thus enhancing the therapeutic effects. But the early data from both of the clinical trials (Kirn et al., 2001) and animal models (Sauthoff et al., 2003) indicated that barriers within the tumor prevent viral spread, which is a major obstacle for achieving clinically relevant therapeutic efficacy. In this chapter, we described a novel approach to enhance the in vivo efficacy of replicating adenovirus therapy by combining a fusogenic viral glycoprotein-induced tumor cell fusion and also showed that the viral therapy can be retargeted by expressing FMG selectively in tumor cells with elevated level of Ras/MAPK via the COX-2 3'UTR element.

Our in vitro studies show that the FMG-induced fusion significantly enhances the oncolytic activity of replicating adenovirus. By ligating the GALV FMG with the COX-2 3' UTR, we were able to regulate GALV-induced tumor cell fusion selectively in cells that have activated Ras/MAPK signaling cascade. In vivo, we demonstrated that the efficacy of intratumoral injection of replicating adenovirus is greatly enhanced by the co-injection of an FMG-encoding plasmid. This combinatorial approach was sufficient to eradicate very small-established tumors at viral doses where injection of the virus, or plasmid alone, was not effective. And, the selective expression of GALV by the COX-2 3'UTR element only in tumor with elevated level of activated Ras/MAPK was sufficient enough to regulate this combined therapeutic approach.

The enhanced therapeutic effects that observed in this combination therapy were not due to increased destruction of the stroma between tumor cells which might act in patient tumors to impede virus spread, because the stroma in these experiments are of murine origin which do not express fusion competent receptors for the GALV FMG. These data suggest that replicating adenoviruses expressing the GALV FMG will be significantly more potent than viruses without FMG. In addition, we have shown that targeting of GALV expression to tumor cells is possible at posttranscriptional levels as demonstrated in this work. This strategies could be combined with pre-existing conditionally replicating adenoviruses which have tumor targeting already built in either at the level of transcriptional regulation using selective promoters driving essential replicative (Rodriguez et al., 1997) (Hallenbeck et al., 1999) or therapeutic genes (Freytag et al., 2002) or at the genetic level using viral mutants with tumor cell selective replicating properties (Alemany et al., 2000).

The results from the in vitro studies to understand the interaction between FMG and adenovirus replication show that the FMG-mediated syncytial formation significantly enhances the efficacy of replicating adenoviral therapy through a combination of mechanisms. One of these is a moderate increase in the transfectability of cells with the GALV plasmid in the presence of co-infecting adenoviruses. This effect will boost the cytotoxicity associated with the FMG-mediated component of the therapy (Bateman, 2002) (Diaz et al., 2000). However, quantitatively more significant, the levels of tumor cell that expressed the viral E1A protein were markedly increased in the presence of FMG-mediated cell-cell fusion. Elevated cellular levels of E1A were associated with significantly increased total viral titers (total virus produced from infected cells) as well as greater viral release from cultures of infected syncytial cultures compared to normal non-fusing

monolayer. Finally, as a result of all of these effects, and consistent with report using the HIV gp120 FMG (Li et al., 2001b), syncytia also promoted enhanced spread of adenoviral particles through the tumor cell cultures. Beside the overall increase in viral titer, syncytial formation also increased release of adenoviral particles. This is most probably due in part at least to lysis of infected cells thereby releasing intracellular particles, which would otherwise remain cell associated. Therefore, the increase level of viral production in combination with the accelerated release of viral particles through tumor cell cultures would suggest that combination therapy with FMG expression and replicating adenovirus infection may overcome some of the problems associated with the use of replicating adenovirotherapy, which have been seen to date.

Chapter 6

A Cell Cycle Dependent Conditionally Replicating Adenoviral Vector for Cancer Gene Therapy.

6.1 Introduction:

We previously showed that successful construction of a conditionally replicating adenovirus to target the RAS-MAPK pathway in the cell can be achieved by the cyclooxygenase-2 3'-UTR. In order to expand our hypothesis and prove that the 3'UTR element from other tumor associated genes can also be used to target tumor cells, in the studies presented in this chapter we used the 318 bp 3'UTR element from the DNA methyltransferase1 gene (DNMT1) and demonstrated *in vitro* that genes can be selectively expressed in rapidly dividing tumor cells. Also, by introducing the DNMT1 3'-UTR in between the adenoviral E1A coding sequence and the poly A signal, E1A expression, and subsequently viral replication, becomes restricted to the tumor cells derived from multiple tissue types.

DNA methyltransferase1 is a major enzyme that is responsible for maintaining the methylation pattern from the parent cell into the daughter cell during cell division. The expression of DNMT1 is tightly coordinated with the DNA replication (Araujo et al., 1999) during cell division (Szyf et al., 1985). Several lines of evidences link DNMT1 with the tumorigenic process. First, increased levels of DNMT1 mRNA, protein and activity were observed in many different types of cancer. Second, overexpression of DNMT1 in the mouse fibroblast NIH3T3 cells caused cellular transformation (Wu et al., 1993). Also the DNMT1 promoter regions contain multiple activator protein 1 (AP-1) elements, which can be transcriptionally activated by proto-oncogene and mitogenic signals (Girault et al., 2003). And finally, downregulation of DNMT1 expression by RNA interference, antisense or pharmacological inhibitor induced demethylation of DNA and re-expression of various tumor suppressor genes (Robert et al., 2003), which lead to inhibition of DNA replication

(Knox et al., 2000) and cell growth (Laird et al., 1995). Thus, DNMT1 has been suggested to play an important role in cancer development.

DNMT1 mRNA levels are significantly downregulated during the G_0/ G_1 phase of the cell cycle, but the abundance of DNMT1 mRNA is dramatically increased after cells enter into the S phase (Robertson et al., 2000; Szyf et al., 1991). The 3'-untranslated region (3'-UTR) of the DNMT1 mRNA plays an important role in the cell cycle-dependent regulation of DNMT1 expression. A 54-nucleotide highly conserved AU-rich element (ARE) within the DNMT1 mRNA is responsible for orchestrating cell cycle-dependent DNMT1 expression by destabilizing its own mRNA in the quiescent cells {Detich, 2001 #3867}. 40 kd protein (p40) interacting with this conserved region of DNMT1 3'UTR may be involved in destabilizing DNMT1 mRNA (Detich et al., 2001). As the cell enters into S phase, the binding of p40 to the 54-nucleotide element is significantly reduced by some unknown mechanisms and the mRNA is restabilized. Because tumor tissues are presumed to contain a greater proportion of dividing cells than their normal counterparts and increased DNMT1 expression is observed in many different tumor samples, targeting cancer via tumor cell specific mRNA stabilization by the DNMT1 3'-UTR could be a viable strategy.

In this chapter we are able to show that DNMT 3'UTR element can be used to regulate transgenes expression selectively in various tumor cells. By inserting this element in the adenoviral genome we were able to control E1A expression and viral replication in vitro. We were also able to demonstrate that DNMT1 3'UTR-mediated regulation of adenoviral replication was correlated with cell cycle dependent DNMT1 protein expression in the A549 cancer cell.

6.2 Results:

6.2.1 DNMT 3'UTR is active in tumor cells

To evaluate tumor cell specific activity of DNMT 3'UTR, a transient transfection of luciferase reporter assay was performed. The luciferase activity of the positive control plasmid with the SV40 enhancer/promoter (Fig. 1A) in each cell line was considered as 100%. As shown in Fig. 1B, the DNMT 3'UTR containing pGL3 construct showed similar luciferase reporter activity compared to the control vector in most of the tumor cell lines tested. In contrast, luciferase activity was significantly inhibited (10-12% of control vector) in the primary human retina pigment epithelial cells hTERT-RPE1 and IMR90. The other primary cell line used in this experiment was BEAS-2B, which is a human bronchial epithelial cell immortalized by SV-40 large T antigen, and showed similar luciferase reporter activity to control plasmid in the presence of DNMT1 3'-UTR. It has been reported previously that SV-40 large T antigen transformed cells overexpressed DNMT-1 during cellular transformation (Chuang et al., 1997) by stabilizing the DNMT-1 mRNA at the posttranscriptional level (Slack et al., 1999). So, SV-40 large T antigen may also stabilized DNMT1 massages in the BEAS-2B cell and responsible for the increased luciferase activity. Western blot analysis of DNMT1 expression in the various tumor and primary cell lines revealed that the DNMT1 expression is significantly elevated in most of the tumor cell lines tested compared to primary cell lines except BEAS-2B cells.

6.2.2 The DNMT1 3'-UTR can selectively down-regulate the stable luciferse mRNA in primary cells but not in tumor cells

To validate the hypothesis that the *cis*-acting sequence within the DNMT1 3'-UTR can stabilize the mRNA levels in rapidly dividing tumor cells, but destabilize the same mRNA in slow growing primary cells, we performed a Northern blot for luciferase mRNA, extracted from transiently transfected human primary cells (hTRE) and tumor cells (HCT116). In the presence of the DNMT1 3'-UTR, steady-state reporter luciferase mRNA expression was significantly inhibited only in the primary cells (**Figure 1C**). In contrast, in tumor cells the DNMT1 3'-UTR did not alter the luciferase mRNA expression. These finding suggested that the DNMT1 3'-UTR possesses a potent inhibitory effects on the reporter mRNA expression only in primary cells, but not in tumor cells.

6.2.3 Construction of AdDNMT virus

Therefore, we constructed AdDNMT, a conditionally replicating adenovirus where the replicative essential viral gene E1A was ligated to the DNMT1-3'UTR using a PCR strategy. Figure 2 depicts the genome of the recombinant adenoviruses. The viral DNA was isolated by Hirt extraction and the modified sequences were confirmed by PCR (data not shown).

6.2.4 AdDNMT induced tumor-specific cytotoxicity

To evaluate the cytolytic activity of the AdDNMT in the context of oncolytic adenovirus, a human colorectal cell line HCT116, and the primary cell line hTRE were infected with AdDNMT or wild type Ad-5 (wt Ad-5) at an MOI of 1. After 7 days, cells were examined for CPE. Figure 4A depicts representative results of selective oncolysis of the AdDNMT virus. As expected, wt Ad-5 virus induced cytotoxicity in both tumor and normal cells

without any specificity. In contrast, the AdDNMT infection induced selective oncolysis in the HCT116 tumor cell line, but no significant oncolysis was observed on the normal hTRE cell line. The morphology of AdDNMT-infected hTRE normal cells appeared to be very similar to the uninfected cells (**Figure 4A**).

To further monitor the oncolytic activity of Ad-DNMT by the quantitative measurement of the cell viability, MTT assays were performed exposing a panel of human primary (hTRE, hEPC, HUVAC and IMR90) and tumor cell lines (HCT116, Mel 88, Mel 624, huh7 and U251) to AdDNMT or wt Ad-5 virus at MOI of 1. In most of the tumor cell lines tested, the oncolytic activity of the AdDNMT virus was as efficient as wt Ad-5 virus, with some differences in the kinetics of cell killing between different cell lines (Fig. 3B and 4B). While greater than 90% of HCT116 and U251 tumor cells were killed within 6-8 days, longer times were required with the Mel 888 and huh-7 cell lines to observe comparable levels of cytolysis. But in all the tumor cell lines, AdDNMT and wt Ad-5 virus showed very similar kinetics of oncolysis. In contrast, in most of the human primary cell lines, AdDNMT showed some toxicity at the earlier time point, but in the later time point AdDNMT induced oncolysis was significantly reduced compared to wt Ad-5 virus. The cytotoxicity observed by AdDNMT infection at the earlier time points in some primary cell lines could be due to the viral particle induced toxicity rather than oncolysis. These findings confirmed that AdDNMT mediates selective oncolysis in tumor cells, but the oncolysis is significantly reduced in normal cells.

6.2.5 AdDNMT mediated tumor cell-specific E1A expression

To ensure the specificity of DNMT1 3'-UTR, human tumor (HCT116) and primary (hTRE) cells were infected with AdDNMT at an MOI of 10. After 48h of incubation, the

expression of E1A protein was evaluated by Western blot. As shown in figure 3C, the expression of E1A in AdDNMT infected HCT116 cells was detectable but lower than wt Ad-5 infected cells. In contrast, E1A expression was completely undetectable in AdDNMT infected primary hTRE cells, thus demonstrating the specificity of DNMT 3'-UTR (Figure 3C).

6.2.6 Cell cycle dependent DNMT1 expression correlate with AdDNMT replication and oncolysis in A549 cells

We observed that the lytic activity of the AdDNMT virus in A549 cells is dependent on the seeding density of the A549 cells at the time of AdDNMT infection. If the cell culture monolayer of the A549 cells reached full confluence during infection, the oncolytic activity of the AdDNMT virus was significantly reduced compared to Wt Ad-5 virus (**Figure 5C-100% confluent**). But if the monolayer was sub-confluent at the time of infection, the oncolytic activity of the AdDNMT virus was very similar to the Wt Ad-5 virus. Immunoblot analysis revealed that DNMT1 expression was significantly lower in the confluent cultures (**Figure 5B**), which also contained fewer number of cells in the S-phase of the cell cycle. Based on these results, we conclude that the oncolytic activity of the AdDNMT virus in A549 cells depend on the percentage of cells are in S-phase of the cell cycle and also correlate with the cell cycle dependent DNMT1 expression.

Figure 1. **Immunoblot analysis for DNMT-1.**

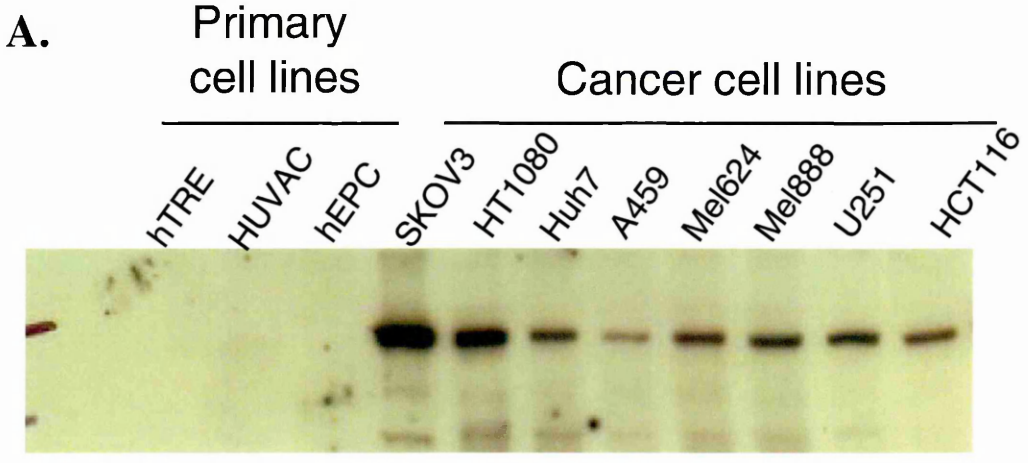
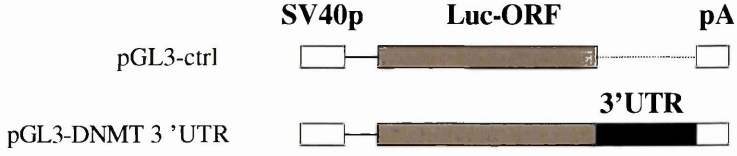


Figure 1 A. Level of DNMT1 expression analyzed by Western blot in the immortalized primary cell lines and different cancer cell lines. Equal amount of protein (15 μg) loaded into each lane.

Figure 1. **The DNMT 3'UTR destabilizes a reporter mRNA in Primary human cells.**

B.



C.

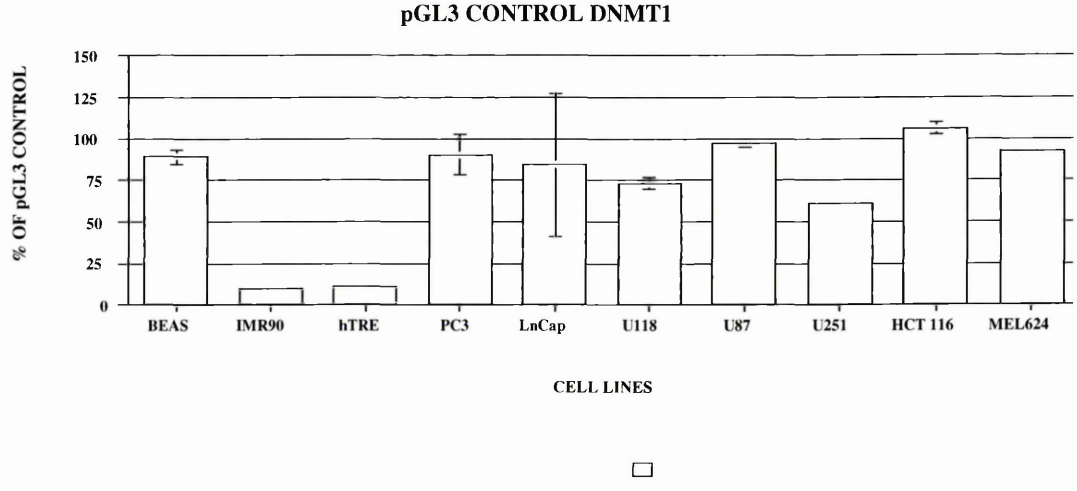


Figure 1. B. Schematic representation of DNMT1 3'-UTR luciferase reporter plasmids. A 318-bp PCR product coding for DNMT1 3'-UTR was inserted between the luciferase reporter gene and SV40 poly A signal to investigate the selectivity of the DNMT1 3'-UTR. **C.** the DNMT1 3'-UTR activity was assessed by luciferase assays. The DNMT1 3'-UTR activity in each cell line was calculated as percentage of the positive control (pGL3-ctrl). The data shown are MEAN ± s.d. (bars) and values (n=3).

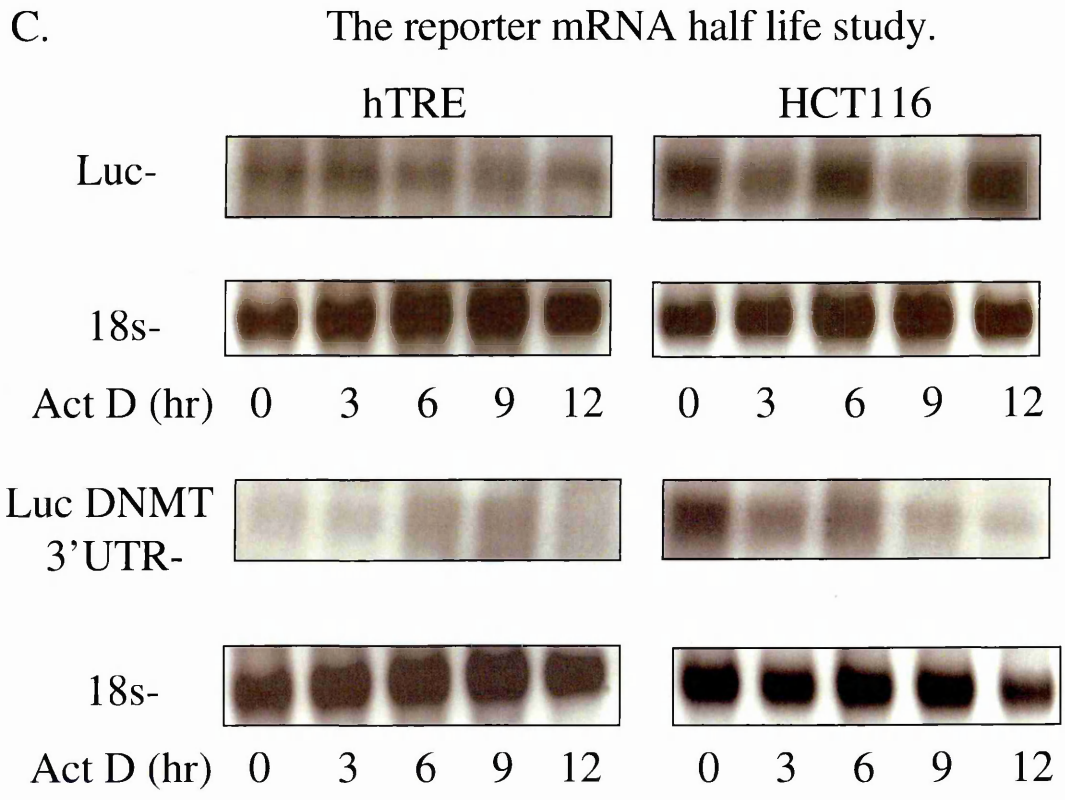


Figure 1. C. Differential DNMT1 3'UTR decay rates *in vitro*. The primary cells hTRE and the tumor cells HCT116 were transiently transfected 1 μg of pGL3 plasmid with reporter luciferase gene and luciferase gene ligated to DNMT 3'UTR. After incubation for 18 hours, cells were harvested (Act D 0h) or further incubated with 5 μg actinomycin D/ml. The cells were harvested every 3 hours for 12 hours and the reporter luciferase mRNA levels were determined by Northern blot analysis.

Figure 2. **Schematic representation of the AdDNMT vector and the the AdDNMT-tk vector genomes.**

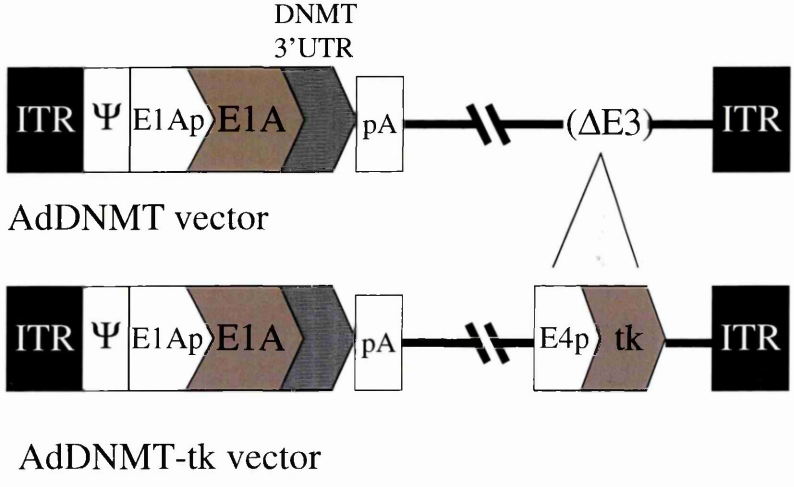


Fig. 2. Schematic representation of the adenoviral vector AdDNMT.

Figure 3. **Cytopathic effects of AdDNMT on human primary and tumor cell line.**

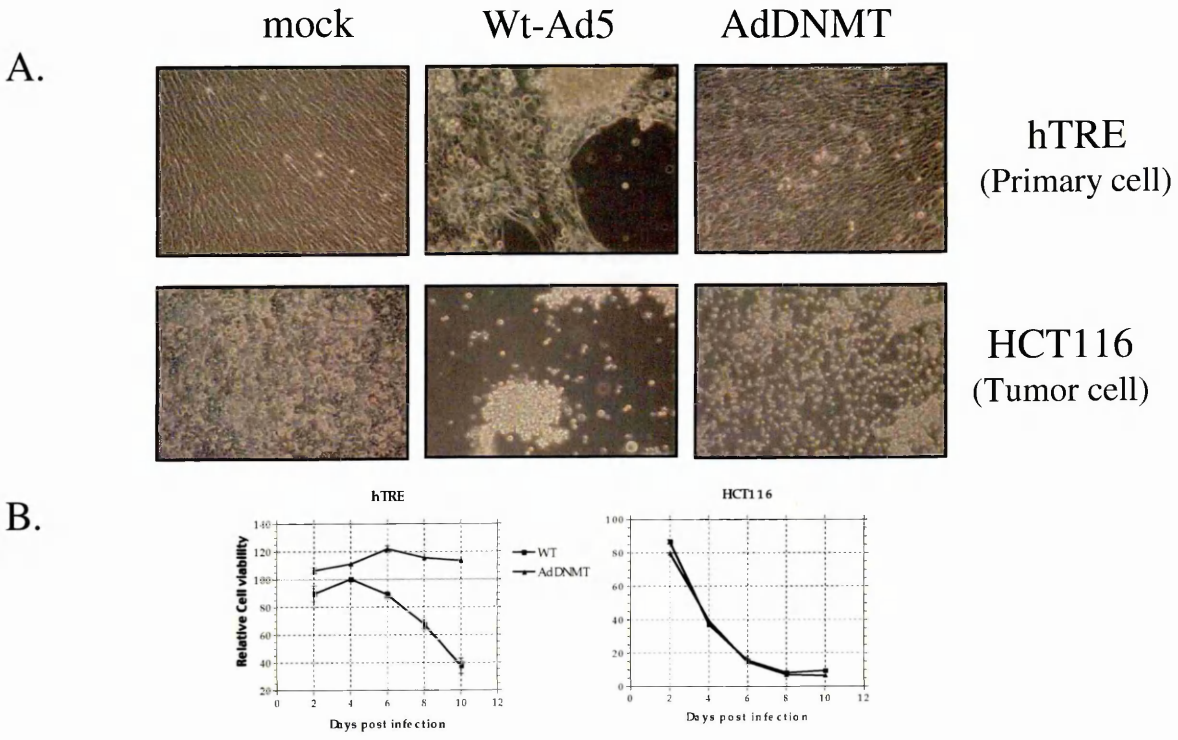


Figure 3C. **Immunoblot analysis for E1A protein.**

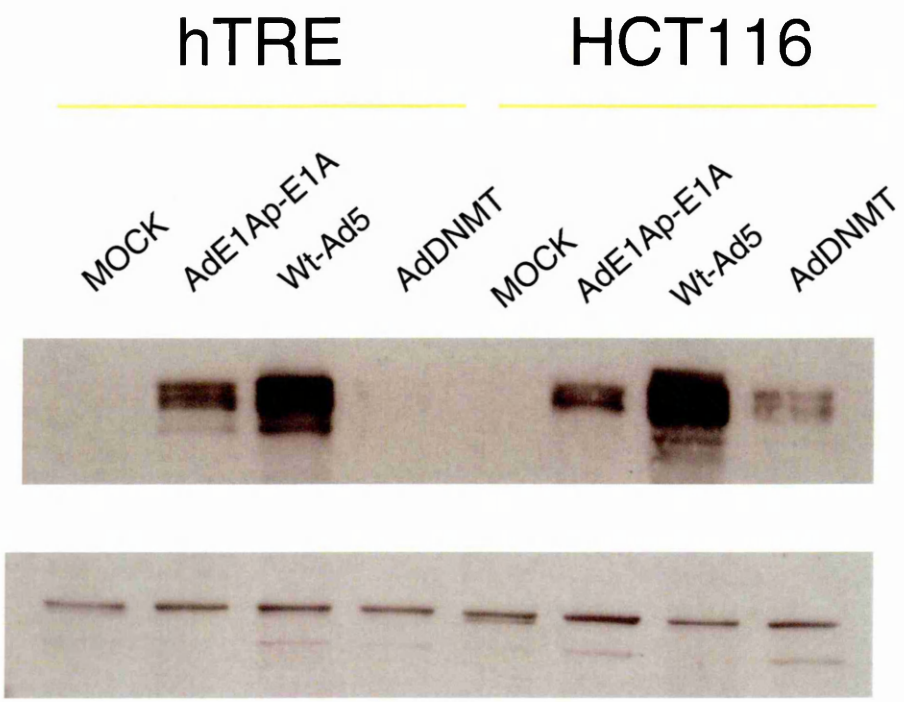
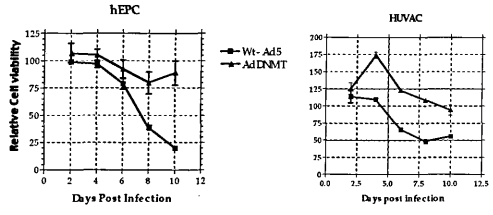


Figure 3. Tumor-selective cytotoxicity of AdDNMT. **A**, subconfluent tumor cells (HCT116) and normal cells (hTRE-RPE) were infected with MOI 1 of AdDNMT or wt Ad5 virus. Mock-infected cells were introduced as control. After 7 days, the appearance of cytopathic effect (rounding and detachement) was monitored, and documented as photographs. **B**, Monolayers of tumor (HCT116) and normal cells were infected with MOI 1 of wt Ad5 (■) or AdDNMT (▲) virus. MTT assay was performed to measure viable cells, and results are the mean of triplicate experiments and expressed as percentage of non-infected cells. C. . Levels of E1A expression in the HCT116 cancer cell lines U87, U118 and U251 were assayed by Western Blot 15 hours following infection with Ad-E1A or Ad-E1A-COX viruses at an m.o.i. of 10. Lane 1 and 5 Mock infected, lane 2 and 6 AdE1Ap-E1A: cells infected with recombinant virus that express E1A gene under E1A promoter,

lane 3 and 7 Wt-Ad5: cells infected with wild type adenovirus 5 and lane 4 and 8 AdDNMT: cells infected with recombilant virusthat express E1A gene under E1A promoter and fused with DNMT 3' UTR.

Figure 4. **Oncolytic effect and tumor specificity AdDNMT vector.**

A. Reduced toxicity of AdDNMT virus on human primary cell lines.



B. Tumor cell-specific oncolysis of AdDNMT virus.

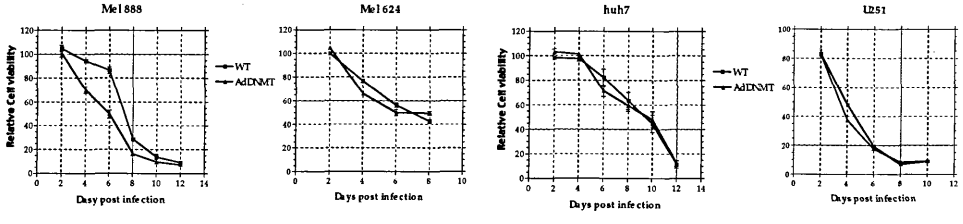


Figure 4. Oncolytic specificity of the AdDNMT vector. Monolayers (**A**) of normal and tumor (**B**) cells were infected with wt Ad5 (■) or AdDNMT (▲) virus at a MOI of 1. MTT cytotoxicity assay was performed at the time points shown. The viability of each infected culture at each time point is expressed as a percentage of uninfected controls. Each data point is the mean of three replicates; bars, ± s.d.

Figure 5. **Cell cycle dependent oncolytic activity of AdDNMT in A549 cell.**

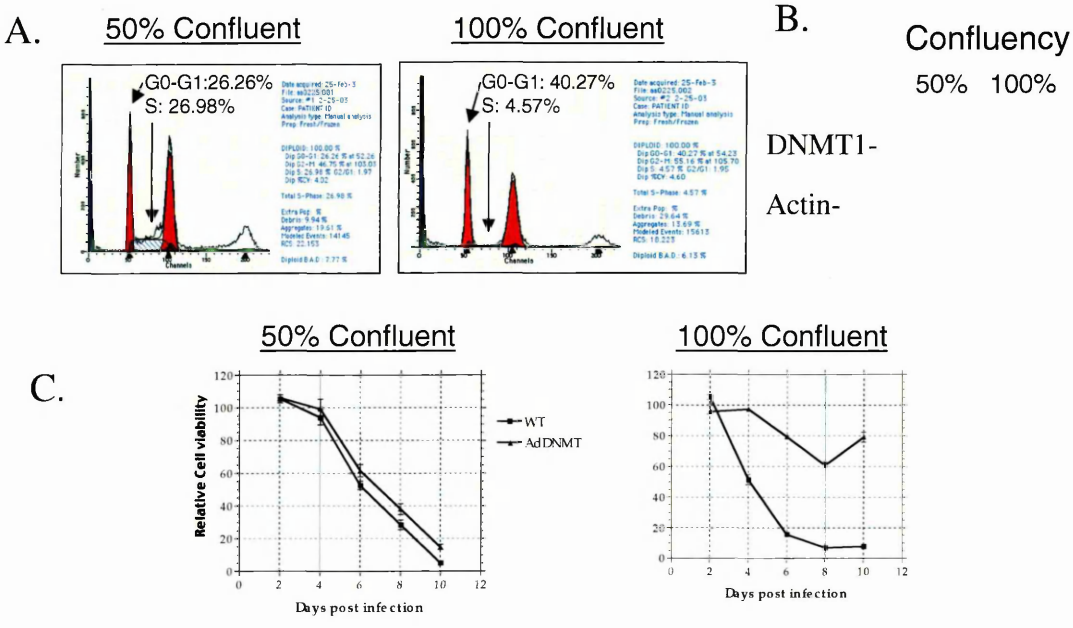


Figure 5. Cell cycle dependent oncolytic activity of AdDNMT in A549 cell. **A.** Cell cycle analysis by flow cytometry of A549 cells plated in different density. **B.** Immunoblot analysis of the DNMT1 protein level in the same population of A549 cells as figure 5A. **C.** Monolayers of A549 cell with different density (as figure 5A) were infected with MOI 1 of wt Ad5 (■) or AdDNMT (▲) virus. MTT assay was performed to measure viable cells, and results are the mean of triplicate experiments and expressed as percentage of non-infected cells.

6.4 Discussion:

The studies presented in this chapter provide *in vitro* evidence supporting the use of tumor cell selective mRNA stabilization by the DNMT1 3'UTR for developing a conditionally replicating adenovirus vector for cancer treatment. Our results demonstrate that (a) the minimal 318 bp DNMT1 3'-UTR can induce tumor cell selective mRNA stabilization of E1A gene to drive adenovirus replication and oncolysis, (b) the 3'-UTR also causes inhibition of the E1A gene expression and adenovirus replication in primary cells derived from different tissues, and (c) AdDNMT replication and oncolysis in the A549 tumor cell line correlate with cell cycle dependent expression of DNMT1.

The overexpression of the DNMT1 mRNA and protein has been reported in many different types of cancers (Etoh et al., 2004; Girault et al., 2003; Nakagawa et al., 2003; Nakagawa et al., 2005; Shieh et al., 2005). It is speculated that in cancers, DNMT1 is involved in the DNA hypermethylation and maintenance of CpG islands that are not methylated in normal cells (Etoh et al., 2004). The ubiquitous expression of DNMT1 in many different types of tumor has made the enzyme an attractive pan-tumor target for developing oncolytic adenovirus.

The DNMT1 3'UTR used in this report was significantly more active in a variety of cancer cell lines with higher expression of DNMT1. By inserting the DNMT1 3'UTR in between the luciferase reporter gene and polyA signal, the luciferase activity was 8 to 10-fold reduced in normal hTRE-RPE cells, which do not express any DNMT1 (**Figure 1**). But in another primary cell line BEAS-2B, the luciferase activity was not controlled by the DNMT1 3'UTR. The BEAS-2B cell line is a normal human bronchial epithelial cell, which is immortalized by SV40 large T antigen. It has been reported that the DNMT1 expression

is a downstream effect of SV40 large T antigen expression and the DNMT1 3'UTR plays an important role in DNMT1 expression in cells that express SV40 large T antigen (Chuang et al., 1997). We believe that the inability to repress reporter gene activity by the DNMT1 3'UTR in the BEAS-2B cells is due to SV40 large T antigen-mediated DNMT1 mRNA stabilization. On the other hand, DNMT1 3'UTR did not affect the reporter gene expression in most of the tumor cell lines that express DNMT1 (**Figure 1A and 1B**). In the oncolytic activity measured by the MTT assay, the wild-type Ad5 virus showed a similar killing potency in normal and cancer cells, thus lacking tumor cell specificity. In contrast, all the tumor cell lines tested, AdDNMT adenovirus induced cell killing as potently as the wt Ad-5 virus, but was approximately 10-50 times less effective in normal cells, confirming tumor cell-specificity of the DNMT1 3'UTR. We were also able to show that in A549 cells, the replication and the oncolytic activity of the AdDNMT virus correlate with the DNMT1 protein expression in cells plated with different density. Thus, the A549 cells plated in higher density became cytostatic by increasing the number of cells in the G_0/ G_1 phase of the cell cycle (**Figure 5A**) in the reducing the number of cells metabolically active (data not shown). Under these conditions, A549 cells downregulate DNMT1 protein expression. The mechanism of DNMT1 downregulation in the confluent A549 cells is not yet understood. But we are able to show that in the confluent cultures of A549 cells, the oncolytic activity of the AdDNMT virus was significantly reduced compared to the control wt Ad-5 adenovirus. In contrast, the semi-confluent A549 cells the oncolytic activity of the AdDNMT virus was identical to the wt Ad-5 correlating the correspondingly high levels of DNMT1. Thus, from these results we can conclude that the novel DNMT1 3'UTR tested in

this study can control transgene expression, and viral replication, in a tumor-specific manner.

A successful viral vector for cancer gene therapy not only needs to demonstrate the therapeutic efficacy by efficiently eliminating all the cancer cells, but also needs to be selective enough to use it systemically. Most of the current strategies for developing cancer gene therapy vector are not tumor-selective enough to fulfill all the safety requirements for systemic use. Major efforts have been directed toward improving or finding new and better tumor targeting strategies to achieve higher degree of tumor selectivity and lower the toxicity caused by therapeutic vectors. The tumor selective mRNA stabilization by the DNMT 3'UTR presented in this study can not only be used as a novel pan-targeting approach to control therapeutic or viral gene expression in tumor cells, but it can also be used for improving the selectivity of currently available tumor targeting strategies. Many tumor specific promoters that are being used for developing oncolytic virus for cancer gene therapies are not entirely selective for tumor cells and generally active in one, or at most, a few normal tissue types. Due to this leakiness, systemic use of the oncolytic vectors developed by these tumor specific promoters is difficult because of the toxicity induced by these vectors. Although tumor cells tend to overexpress DNMT1, it has been reported that some proliferating normal cells constitutively express DNMT1 (Leonhardt et al., 1992). The difference in the mechanisms involved in controlling the expression of DNMT1 in the normal proliferating cells and cancer cells is not well understood, but it is a possibility that the AdDNMT virus may replicate and show some toxicity in these normal cells. We believed that the combination of tumor specific mRNA stabilization mechanisms of the DNMT 3'-UTR with a tissue specific promoter could be a solution for developing a safer

oncolytic vector. The tumor cell specificity of different tumor specific promoters can be enhanced by adding the translational specificity of DNMT1 3'UTR, which should help each other to express therapeutic gene more selectively in the tumor. The data presented here suggest that DNMT 3'UTR mediated tumor specific mRNA stabilization could be use as a means to achieve tumor-specific expression of therapeutics, or viral genes, for cancer gene therapy.

Chapter 7

Discussion and future direction

In this thesis, the hypothesis to be tested is that tumor cell selective stabilization of mRNA can be used to control therapeutic/viral gene expression in order to develop targeted viral vectors for cancer gene therapy. The data presented indicates that linkage to the AU-rich 3' untranslated region (UTR) from a tumor-associated gene, cyclooxygenase-2 (COX-2), of a replicative essential viral gene is sufficient to achieve tumor cell selective viral replication and oncolysis.

The expression of COX-2, which has been associated with poor prognosis in many different types of tumor and is a downstream effect of RAS-mediated transformation (Sheng et al., 2001). Upregulation of COX-2 in the transformed environment is partly mediated by a selective stabilization of the COX-2 mRNA (Sheng et al., 2000) (Dixon et al., 2000) and the AU-rich region of the 3'UTR of COX-2 mRNA is essential for stabilizing the COX-2 mRNA in an activated RAS-MAPK-dependent manner (Lasa et al., 2000). So, we hypothesized that tumor selective transgene expression can be achieved by using an AU-rich stabilization element such as the COX-2 3'UTR in a viral vector for cancer gene therapy. Our hypothesis relies on the proven ability of the COX-2 3'UTR region to destabilize a message in the quiescent environment, but restabilize the same mRNA in the presence of the appropriate oncogenic stimuli.

The initial studies were designed to clone the AU-rich element from the 3' UTR of the tumor-associated gene COX-2 and evaluate it's the ability to regulate gene expression in transformed and non-transformed cells. 469 base pairs of the COX-2 3'UTR was cloned (Dixon et al., 2000) and inserted downstream of the replicative essential adenoviral gene E1A, which was driven by the human CMV to generate the CMV-E1A-COX construct. The effect of the COX-2 3'UTR insertion on E1A expression was characterized in a model

system where exactly matched rat intestinal epithelial cell lines (RIE-iRAS), differing only in the expression of an activated inducible variant of the H-Ras oncogene were used (Sheng et al., 2000). Plasmid transfection of the adenoviral E1A gene, with or without, the COX-2 3'UTR in the RIE-iRAS cells, followed by infection with an E1A-deleated, replication-incompetent adenoviral vector expressing GFP reporter gene (Ad-GFP) highlighted a number of points. First of all, transient expression of E1A (CMV-E1A) in RIE-iRAS cells was able to support the replication and mobilization of the incoming Ad-GFP vector, which was independent of the inducible expression of Ras oncogene. In contrast, cells transfected with CMV-E1A-COX plasmid were able to support replication and mobilization of the Ad-GFP vector only when RIE-iRAS cells were previously induced to express activated Ras. Secondly, experiments designed to assess viral titers showed a clear difference between the titer of Ad-GFP released with, or without, inducible RAS signaling in the RIE-iRAS cells transfected with CMV-E1A-COX plasmid. And finally, inhibition of Mitogen-activated protein kinase (MAPK) activation, a downstream effectors molecule for Ras signaling cascade, greatly reduced the ability of CMV-E1A-COX to support Ad-GFP replication and mobilization even in the presence of inducible activated Ras. From these experiments, we were able to conclude that, at least *in vitro*, the COX-2 3'UTR is able to control gene expression, which was highly dependent on oncogenic Ras signaling.

To test whether the regulation of gene expression by the COX-2 3'UTR is sufficient to control viral replication, we incorporated the CMV-E1A and CMV-E1A-COX cassettes into E1A-deleted adenoviral genomes. The production of recombinant adenovirus expressing these cassettes was performed using standard methods and the recoverable titer of adenovirus expressing CMV-E1A (Ad-E1A) or CMV-E1A-COX (Ad-E1A-COX) was

very similar to the wild type Ad-5 titer. The replication selectivity of the Ad-E1A and the Ad-E1A-COX virus were tested in the model cell culture system RIE-iRAS cells. Replication and oncolysis of the Ad-E1A-COX virus was heavily dependent upon the expression of the inducible activated Ras and consistently produced an excess of 3 log more viruses compared to the same cultures without any inducible activated Ras. Ad-E1A-COX virus replication in the Ras induced RIE-iRas cells was also inhibited (about 2 log inhibition) by blocking MAPK activation with a chemical inhibitor, which again validated the requirement for activated Ras/MAPK intracellular signaling in COX-2 3'UTR mediated regulation of gene expression. We were also able to show that the oncolytic activity of the Ad-E1A-COX virus in different human tumor cell lines correlated very closely with the cell lines' MAPK activity status. *In vivo* in two human tumor xenograft models with elevated levels of activated MAPK (U87 and U251), the Ad-E1A-COX virus showed similar therapeutic efficacy as the wild type Ad-5 virus, but showed no significant therapeutic effects in the tumor model with very minimal activated MAPK (U118). Taken together, these data suggest that E1A expression can be regulated in an adenoviral genome by the COX-2 3'UTR and Ad-E1A-COX virus is oncolytic both *in vitro* and *in vivo* against human tumors with elevated level of activated RAS-MAPK signals.

Results from the toxicity and the biodistribution studies in the immunocompetent murine model show that the systemic administration of Ad-E1A-COX virus induces some hepatic toxicity compared to the wild type Ad-5 virus. Although the lethal dose 50 (LD50) for both of the viruses were very similar, the mice treated with Ad-E1A-COX virus had elevated levels of liver enzyme in the blood and showed abnormal liver histopathology with increased immune infiltrate. This toxicity was not associated with the organ-specific

non-selective replication of the Ad-E1A-COX virus because the amount of virus recovered from the liver of the mice treated with the Ad-E1A-COX were lower than the mice treated with the wild type Ad-5 virus. However, at the early time point (24 h post i.v. challenge) we observed an increased amount of viral DNA and E1A protein in the livers of the Ad-E1A-COX treated group compared to the wt Ad-5 group. It is possible that this early elevation of viral DNA and protein could trigger a stronger innate immune response, which then would lead to hepatic toxicity. It is known that during cell entry, the interaction between the adenoviral entry receptors (fiber knob and penton base (Roelvink et al., 1998)) and the cellular entry receptors (CAR and $\alpha_V\beta_3$ and $\alpha_V\beta_5$ (Wickham et al., 1993)) activates various intercellular signaling pathways including the MAPK signaling cascade (Bruder et al., 1997) (Tibbles et al., 2002). Based on this, one working hypothesis is that the activation of a MAPK pathway during the initial adenoviral interaction with the cellular entry receptors might alter the resting cell microenvironment and therefore even hyper-stabilize the viral E1A protein ligated to the COX-2 3'UTR. This hypothesis is recently supported by Crofford et al, showing that the initial interaction between adenovirus and liver synoviocytes is sufficient to increase COX-2 expression both at the mRNA and protein levels, and MAPK signaling is vital for this COX-2 induction (Crofford et al., 2005). Taken together, results from the initial in vivo toxicity show that the 3'UTR needs to be selected very carefully in order to develop tumor selective conditionally replicating viral vectors. The 3'UTR from different genes will have different restrictions, which will depend on the level of expression of that gene in the tumor microenvironment and the expression profile in the normal physiological condition (Derrigo et al., 2000). Also, the kind of vector used to target tumor will be important for the selection of the 3'UTR

elements. It will be probably wise to avoid genes involved in inducing innate immune response for developing retargeted adenoviral vectors, because adenovirus is known to induce potent innate immune responses (Muruve, 2004), which may compromise the selectivity of the 3'UTR element. Recently, several reports show that in order to develop safe and less toxic adenoviral vectors for cancer therapy, multiple viral genes expression need to be regulated (Irving et al., 2004). One possible way to reduce the Ad-E1A-COX induced liver toxicity after systemic administration will be to use the COX-2 3'UTR element to regulate multiple adenoviral genes. For example, the adenoviral late transcription unit will be a very attractive candidate for this targeting approach as the late transcription unit encodes approximately 15-20 different mRNAs, all of which derive from a single pre-mRNA by differential splicing (Young, 2003). Most of the late gene products are viral structural proteins and essential for virion assembly (Rux & Burnett, 2004). If an mRNA stabilizing element, such as COX-2 3'UTR, can be inserted down stream of the late transcription unit, all of these late gene products can be regulated by one single tumor selective element and may lead to improved tumor selectivity and reduced toxicity.

To our knowledge, this is the first example of achieving tumor-selective gene expression, and development of a conditionally replicating adenovirus, through a tumor specific post-transcriptional mRNA stabilization element. There are several issues that need to be addressed in order to optimize post-transcriptional targeting strategies for more efficient tumor targeting. First of all, we have no knowledge about the minimal level of activated RAS-MAPK signaling required for achieving sufficient stabilization of the transgene mRNA in order to target tumor cells through the COX-2 3'UTR. This will give us a clearer idea about the kind of cancer that can be targeted with the Ad-E1A-COX vector. One

possible way to investigate this will be using a more sensitive inducible system such as repaying regulated, where transgene can be expressed in a dose-responsive manner (Pollock et al., 2002) and use these systems to generate stable cell line where constitutively activated Ras or MAPK expression can be more tightly controlled. Also, the receptor tyrosine kinase (RTK) family of proteins such as epidermal growth factor receptor (EGFR) is an upstream signaling molecule of Ras/MAKP signaling cascade and plays an important role in cancer pathogenesis. Several reports postulate a link between the EGFR upregulation and subsequent COX-2 induction in different cancers (Yuan et al., 2005) and the activation of EGFR signaling pathway can induce COX-2 expression by stabilizing the COX-2 mRNA (Matsuura et al., 1999). So the RTK overexpressed tumor cell can be a suitable target for the Ad-E1A-COX virus and we need to test the level of different RTK expression in different cancer cells and try to correlate with the oncolytic selectivity of Ad-E1A-COX virus. Second of all, we did not optimize the length of the COX-2 3'UTR element needed to achieve the maximum levels of tumor selectivity. The 469 bp COX-2 3'UTR used in this study was based on the published data by Dixon et al (Dixon et al., 2000) who reported the position of the six AU-rich sequence motifs (AUUUA) in the 469 bp of the COX-2 3'UTR. Several other AU-rich motifs located outside of the 469 bp COX-2 3'UTR have also been identified. It will be logical to include the rest of the 1000 bp AU-rich motifs from the COX-2 3'UTR in our tumor targeting strategy to see whether we can improve the tumor selectivity of this element. And finally, what kind of viral vector will be the best fit for these pos-transcriptional targeting strategies that will give us the maximum control and tumor selectivity for viral replication. To use mRNA stabilization as a mean to control viral gene expression and tumor selective viral replication, it is possible that

oncolytic RNA viral vectors such as vesicular steatites virus (VSV) or measles virus may be better than DNA viruses because the whole viral RNA genome could be restabilized or destabilized by the 3'UTR element and that may enhance the selectivity of tumor cell specific viral replication. But the most challenging thing for using the RNA virus in this targeting approach will be the production of high titer recombinant vectors. It may be necessary to alter the producer cell by introducing the appropriate intercellular signals such as activated Ras/MAPK signaling, so the viral genome can be stabilized in the producer cell and will allow to generate high titer therapeutic viral vectors.

In order to expand the tumor targeting strategy using selective mRNA stability with other tumor associated genes, we cloned a second 3'UTR element from the DNA methyltransferase 1 (DNMT1) gene, an important enzyme responsible for maintaining the cellular epigenetic pattern from parent cell to daughter cell during cell division (Chen & Li, 2006). Several studies have linked this enzyme with tumorogenesis (Szyf, 2002). Increased DNMT1 expression in various transformed environments is associated with abnormal cell cycle (Szyf, 2001) and increased rate of cell division in tumor cells as compared to their normal counterparts. In dividing cells, the stability of the DNMT1 mRNA is dramatically increased after the cell entry into the S phase and the 3'UTR of the DNMT1 mRNA is crucial for this cell cycle dependent increased mRNA turnover (Detich et al., 2001) (Robertson et al., 1999). Because most cancerous tissues contain a higher percentage of dividing cells compared to normal tissues (Robertson et al., 2000), we thought it might be possible to express therapeutic/viral genes selectively in rapidly dividing tumor cells by using the DNMT1 3'UTR. By inserting the DNMT1 3'UTR element downstream of adenoviral E1A gene we showed that the E1A gene can be expressed selectively in cancer

cells and a recombinant adenovirus (AdDNMT) generated by using the E1A-DNMT 3'UTR construct was able to replicate selectively in various tumor cells. The oncolytic activity of the AdDNMT virus was significantly reduced in three immortalized primary cell lines in the tissue culture. The oncolytic activities also correlated with DNMT1 protein expression and the percentage of cells that were in the S phase of the cell cycle. These *in vitro* data support the conclusion that the DNMT1 3'UTR is able to control transgene expression and adenoviral replication selectively in tumor cells. Currently we are evaluating the selectivity of the AdDNMT virus in the *in vivo* animal model.

One of the major obstacles to effective therapeutic use of replicating viruses as anti-cancer agents is the poor intra-tumoral spread of the released virus where other cell types, cell matrix and areas of necrosis exist (Sauthoff et al., 2003). Even after local administration into the tumor, the virus usually gets trapped between various compartments composed of these different cell-types (Heise et al., 1999b). To overcome this problem, we exploited a viral fusogenic membrane glycoprotein (FMG), which is a cytotoxic gene that induces cell killing through induction of tumor cell fusion by forming large multinuclear syncytia. Our laboratory previously demonstrated that the gene transfer of FMG in a xenograft tumor model inhibits tumor growth (Bateman et al., 2000a). A recent report also showed that the HIV gp120 -mediated syncytia formation facilitates the dispersal and increases the infection efficiency of an adenoviral vector in vitro (Li et al., 2001b). Also, an oncolytic herpesvirus expressing Gibbon Ape Leukemia Virus (GALV) hyperfusogenic FMG enhances the therapeutic efficacy of the oncolytic virus in vivo (Fu et al., 2003). Based on these results, we hypothesized that the FMG expression during viral replication inside the tumor might enhance the therapeutic efficacy of the oncolytic viral therapy by

increasing both the release of the viral particles from the infected cells and the spread of the viral particles through out the tumor via the syncytia. We were able to show that GALV induced syncytia enhances viral titer and spread of adenoviral vector through a monolayer. In the presence of GALV induced syncytia, the titer of the virus released into the culture medium is two logs higher than without syncytia. Increased viral titer in syncytia was associated with the upregulation of adenoviral E1A protein expression through unknown post-transcriptional mechanisms. We do not know whether the FMG-induced syncytia affect the expression level of the other viral proteins both in early and late phases. It has been previously reported by our laboratory that transgene expression can be upregulated in syncytia culture due to the increased metabolic activity induced by syncytia formation, which can convert the syncytia into transient protein production factories (Bateman, 2002; Higuchi et al., 2000). We were also able to show that a combination of different doses of FMG gene therapy with replicating adenovirotherapy leads to regression of both small and large established tumor xenografts. Moreover, this combined therapy can be effectively targeted to cells expressing activated oncogenic signals (RAS/MAPK) by ligation of the FMG to the 3' untranslated region of the COX-2 gene. We believe that FMG expression needs to be regulated transcriptionally, or post-transcriptionally, in order to avoid toxicity. For safety purpose, it may be wise to keep the FMG expression separate from virus replication, but it could be difficult to get approval from the regulatory committee to use two separate therapeutic arms (intratumoral FMG+ adenoviral therapy) as a therapy for cancer. Therefore, I proposed to develop a conditionally replicating adenovirus expressing FMG selectively in the tumor. In addition, we have no notion about the time, or amount, of FMG expression required for the maximum interaction between the viral life cycles and the

FMG-induced syncytia, which will give us the optimal synergistic enhancement of the therapeutic efficacy. These questions can be addressed by developing a replicating adenovirus that expresses FMG at different stages of the viral life cycle by using different internal viral promoter (FMG expresses at the early phase of the viral life cycle by the E1A promoter or expresses at the late phase of the viral life cycle by using the viral major late promoter (Wills et al., 1994)). Taken together, we used FMG-induced syncytia to enhance the in vivo therapeutic efficacy of an oncolytic adenovirus for the treatment of cancer. The FMG-induced syncytia not only act as a potent cytotoxic and immunostimulatory gene for cancer gene therapy, but also improve oncolytic viral replication, intratumoral spread and production, which enhance the oncolytic properties of the conditionally replicating adenovirus.

The application of gene therapy in the treatment of diseases like cancer is currently limited due to our inability to efficiently target systemic metastasis. Because different types of cancer have diverse biological properties, and since even the most efficient targeting strategy currently available has its limitations, it is unrealistic to imagine that an individual targeting approach will eventually emerge as the most efficient way to target cancer. Besides, most of the tumor specific elements currently used to target cancer cells have proven to be leaky due to their expression in the normal anatomical site (Zeh & Bartlett, 2002). Moreover, most tumors contain a very heterogeneous population of transformed cells (Bignold, 2003) (Khong & Restifo, 2002). Targeting cancer with an individual tumor specific element will create selection pressures, which will drive the cancer to become resistant to therapy (Konson et al., 2004). So, a successful cancer gene therapy approach will likely rely on the ability to combine different targeting strategies to create highly

specialized "mosaic" vectors that safely incorporate different targeting strategies to address specific features of each type of cancer (Borovjagin et al., 2005). We think that the post-transcriptional targeting strategy of using the 3'UTR will be most efficient in combination with other tumor targeting approaches such as transcriptional or transductional targeting. One unique feature of the tumor selective mRNA stabilization element such as COX-2 3'UTR is that we can regulate individual gene expression both at the transcriptional and post-transcriptional levels. This may help us target systemic cancer more selectively and reduce toxicity from the therapeutic viral vectors.

In summary, this thesis described tumor cell selective mRNA stabilization as a novel means to achieve tumor specific therapeutic gene expression. By using two different 3'UTR mRNA stability elements, we were able to regulate three different transgenes selectively in tumor cells. Incorporation of the COX-2 3'UTR in the adenovirus genome was sufficient enough to control viral replication and oncolysis both in vitro and in vivo. Many other genes have been reported to use tumor cell selective mRNA stabilization as a mean to control their expression in the tumor microenvironment. Therefore, targeting cancer through tumor cell selective mRNA stabilization could be a viable strategy, which may have broad applicability and potential in vector development for cancer gene therapy.

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